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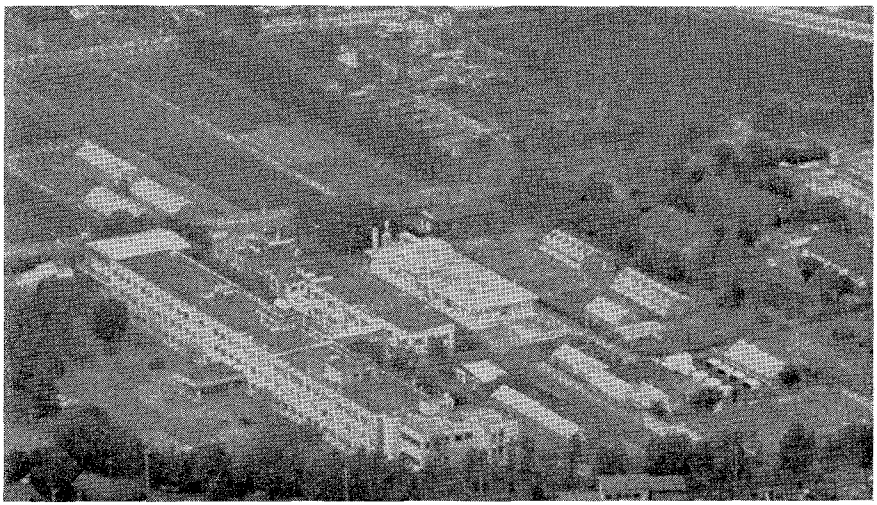
No. 28, 1977



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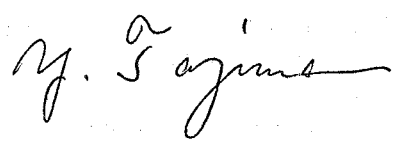
GENERAL STATEMENT

At a request of the Ministry of Education I delivered a lecture on the present situation of recombinant DNA research in the presence of H. I. H. the Crown Prince at the Palace on April 1st, 1977. Introducing my own studies on planned chromosome translocation in the silkworm, I talked on recent advances of genetic studies that had made possible to recombine DNA molecules of different species origin, its anticipated contribution to the advancement of both basic and applied sciences, its conjectured potential hazards to plants and animals including man, and the necessity of guidelines for the safe conduct of the research. My talk was so often interrupted by the Prince's keen questions that the scheduled time was largely exceeded. H. I. H. showed interests not only in biological aspects, but also in the present situation of this research in advanced countries, particularly with regard to obtaining public consent for the promotion of the research. I was very much impressed with the Prince's earnestness. I also talked about the Academy Forum of the U.S. Academy of Sciences held in early March in Washington, D.C., which I participated in. I also referred to the present status of the deliberation on this issue at the Science Council of Japan, expecting that they would reach conclusion by autumn. Indeed, the Council adopted at its 74th general assembly held in October, 1977, a statement that the recombinant DNA researches should be promoted in Japan, taking every possible care of the safety of research workers as well as of the public.

It is my great regret to report here that Dr. Alice Flora Lilienfeld, an honorary member of the Institute, passed away on July 14th, 1977. She was 91 years old. She first came to Japan in 1929, and served as a lecturer at the Faculty of Agriculture, Kyoto University, helping Professor H. Kihara. During the World War II she returned to the United States to avoid the ravages of the war. After the peace was restored, she came again to Japan in 1950 by the invitation of the Kihara Institute for Biological Research. Since then she affiliated also with the National Institute of Genetics as a foreign researcher. As she was proficient in several languages, she took, beside her own research on genetics of *Medicago*, charge of reading the almost all papers written in English or German by the staff members. In this regard we were greatly indebted to her.

Next, I should like to mention a few lines about the organization of the Institute. The Institute was established in 1949 as a national center of the study of genetics under the jurisdiction of the Ministry of Education, when any inter-university research institutes did not exist. The Institute has been independent from any universities, and research staffs have been appointed from qualified scientists in disregard of their academic affiliations. In the meantime several inter-university research institutes have been established with the aim of common use of big research facilities and carrying out cooperative researches. The administrative systems of those institutes are especially suitable for cooperative researches. They can also take part in the education of post graduate students. Our Institute is not granted such privileges. I think it is high time for us to consider seriously whether we should reorganize the system as one of the inter-university research institutes or keep the Institute as it is.

Completed with three laboratories, i.e., plant, animal and microbial, the Genetic Stock Center has already started to carry out its task. The budget for erecting the main building of the Center was approved this year, and the construction work is now under way on the site recently purchased from the City. No wonder, the Center will contribute to the further development of the genetic researches as well as the biological sciences in broad sense. Taking this opportunity I wish to express my deep gratitude to the authorities concerned for their thoughtful and sympathetic assistance.



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OSHIMA, Chozo, Manager, Head of the Physiological Genetics Department

PROJECTS OF RESEARCH FOR 1977

Department of Morphological Genetics

Genetic studies on insect cells in tissue culture (KURODA and MINATO)
Developmental genetic studies on carcinogenesis in tissue culture (KURODA)
Genetics of somatic mammalian cells in culture (KURODA and MINATO)
Cytogenetics in the silkworm (MURAKAMI and IMAI)
Studies on recombination in the silkworm (MURAKAMI)
Genetic studies of the silkworm (TAZIMA and ONIMARU)
Radiation and chemical mutagenesis in the silkworm (TAZIMA and ONIMARU)

Depatment of Cytogenetics

Studies on chromosomal evolution in rodents (YOSIDA)
Chromosome study on experimental tumors (YOSIDA)
Experimental breeding and genetics of mice, rats and other wild rodents (YOSIDA and MORIWAKI)
Cytogenetical study on sister chromatid exchange in mammalian cells (KATO)
Cytogenetical and immunological studies on mouse plasma cell tumor (MORIWAKI)
Biochemical studies on serum transferrin variations in rodents (MORIWAKI)
Immunogenetical study on the histocompatibility antigens of wild rodents (MORIWAKI)
Cytogentical study of ants (IMAI)

Department of Physiological Genetics

Behavior genetics of *Drosophila* (OSHIMA and WATANABE)
Analysis of deleterious and inversion chromosomes in natural populations of *Drosophila melanogaster* (WATANABE)
Studies on fitness of *Drosophila* under controlled environment (OSHIMA and WATANABE)
Genetic studies on the effects of adverse environments on *Drosophila* flies (OSHIMA and WATANABE)

Department of Biochemical Genetics

Studies on transformation in higher organisms (NAWA and YAMADA)
Genetical and biochemical studies of pteridine metabolisms in insects (NAWA)
Analysis of gene action on cell differentiation in higher organisms (NAWA and YAMADA)
Biochemical studies on the differentiation of muscle proteins in animals (OGAWA)
Genetical and biochemical studies of human serum proteins (OGAWA)
Genetical and biochemical studies on Japanese middle size dog (OGAWA)
Genetics of isozymes in plants (ENDO)
Effects of exogenous DNA on plant seed formation (ENDO)
Genetic analysis of developmental mechanisms in hydra (SUGIYAMA and FUJISAWA)

Department of Applied Genetics

Quantitative genetic studies in poultry (KAWAHARA and FUJISHIMA)
Genetics studies in wild populations of Japanese quails (KAWAHARA)
Theoretical studies on breeding techniques (IYAMA)
Behavioral genetic studies in mice (FUJISHIMA)
Genetic studies in natural stands of forest tree species (IYAMA)
Evolutionary studies in wild and cultivated rice species (OKA and MORISHIMA)
Ecological genetic studies in some grass species (MORISHIMA)
Genic analysis for isozyme variations in rice (ENDO and OKA)
Genetic effects of environmental pollution on plants (IYAMA, MORISHIMA and OKA)

Department of Induced Mutation

Molecular mechanisms of spontaneous, chemical- and radiation-induced mutations (KADA, SADAIE and INOUE)
Environmental mutagens and carcinogens (KADA, SADAIE, TUTIKAWA and HARA)
Radiation genetics in mice (TUTIKAWA)
Biochemical factors involved in cellular repair of genetic damage (INOUE and KADA)
Mechanisms of recombination repair (SADAIE and KADA)

Mutation and differentiation studies of plant tissue culture (AMANO and KADA)
Radiation and chemical interaction in the cells (KADA)
Genetic fine structure analysis in maize (AMANO)

Department of Human Genetics

Genetic and epidemiologic studies on certain malformations in human embryos (MATSUNAGA and SHIOTA)
Genetic studies on retinoblastoma (MATSUNAGA)
Clinical cytogenetic studies of congenital abnormalities in man (NAKAGOME and OKA)
Molecular cytogenetic studies of human chromosomes (NAKAGOME)
Studies on human chromosome variants (NAKAGOME, MATSUNAGA and OKA)

Department of Microbial Genetics

Genetic regulatory mechanism of DNA replication in *E. coli* (HIROTA, NISHIMURA, and YASUDA)
Genetic regulatory mechanism of cellular division in *E. coli* (HIROTA)
Molecular genetics on DNA replication (YASUDA and HIROTA)
Molecular mechanisms of cell division in *E. coli* (SUZUKI, NISHIMURA and HIROTA)
Genetics of bacterial cell envelope (HIROTA, SUZUKI, and NISHIMURA)
Synthetic bacterial plasmid (YASUDA, and NISHIMURA)
DNA replication origin of *E. coli* (YASUDA, and HIROTA)

Department of Population Genetics

Theoretical studies of population genetics (KIMURA, MARUYAMA, OHTA, and TAKAHATA)
Studies on molecular evolution from the standpoint of population genetics (KIMURA and OHTA)
Theoretical studies on the evolution of multigene family (OHTA)
Mathematical studies on the genetics of structured populations (MARUYAMA)

Department of Molecular Genetics

Studies on the chemical structure of genome of viruses containing double-stranded RNA (MIURA, SUGIURA and SHIMOTOHNO)

Studies on the interaction between RNA polymerase and template nucleic acid (MIURA, SUGIURA, SHIMOTOHNO, and SOEDA)

Studies on the primary structure of DNA (SOEDA and MIURA)

Studies on structure and function of messenger RNA (MIURA and SHIMOTOHNO)

Genetical and enzymological studies on *E. coli* RNA polymerase (SUGIURA)

Genetic Stocks Center

Studies and conservation of germplasm resources in rice and wheat species (FUJII, SANO and OKA)

On the sex expression in monoecious plants (FUJII)

Specificity of mutagen tolerance in higher plants (FUJII)

Exploitation of genetic ability of nitrogen fixation in Graminae (FUJII, SANO, IYAMA and HIROTA)

Studies on genetic differentiation in rice (SANO)

Studies on teratocarcinogenesis in mice (NOGUCHI)

RESEARCHES CARRIED OUT IN 1977

I. MOLECULAR GENETICS

Inhibition of Messenger RNA Activity by Methylated Guanylic Acid Derivatives

Kin-ichiro MIURA, Yaeko KODAMA and Kunitada SHIMOTOHNO

Most eukaryotic mRNAs carry a "cap" at the 5′-terminal as $m^7G^{5'}ppp^{5'}N(m)$–. Since protein synthesis proceeds in the 5′→3′ direction of the template mRNA, the 5′-terminal modification may be related to the initial step of protein synthesis. The cap ($m^7G^{5'}pp$) can be deleted by tobacco phosphodiesterase with no damage to the RNA chain. The ability to synthesize protein and the ability to form the complex for the initiation of protein synthesis in the cap-deleted mRNA are decreased remarkably (Annual Rep. No. 27 (1976) p. 20; Shimotohno, K., Kodama, Y., J. Hashimoto and K. Miura, Proc. Natl. Acad. Sci. USA, **74**: 2734 1977)). This experiment suggests that the role of the cap is in protein synthesis, especially in the initiation step. Since the cap-eliminated mRNA loses its stability in cell extracts (Annual Rep. No. 27 (1976) p. 20; Shimotohno, K., Kodama, Y., J. Hashimoto and K. Miura, Proc. Natl. Acad. Sci. USA, **74**: 2734 (1977)), the cap may function in protein synthesis through stabilization of mRNA. However, there are some data suggesting that the cap interacts with cellular components required for protein synthesis. Several reports have described that the addition of pm^7G to the protein synthesizing system causes strong inhibition. To determine if pm^7G is specific inhibitor of protein synthesis, various methylated derivatives of guanylic acid were synthesized chemically and their inhibitory effects were studied in wheat germ and *E. coli* cell-free systems.

The effect of adding 7-methylguanylic acid to the *in vitro* protein synthesizing system was studied by incorporation of labeled amino acids into protein in the wheat germ cell-free extract with eukaryotic mRNAs, tobacco mosaic virus RNA and cytoplasmic polyhedrosis virus mRNA. Protein synthesis with both eukaryotic mRNAs was inhibited by 7-methylguanylic acid at concentrations higher than 0.5 mM. Prokaryotic mRNA, MS 2

phage RNA, was not inhibited. The confronting nucleotide structure m^7GpppA, the capping structure at the 5′-terminal of eukaryotic mRNA, was also inhibitory. However, GpppA and GppA, which do not contain a methyl group at the 7-position of G, were not inhibitory.

Since the methylation of guanosine in the cap of mRNA seems to be required for inhibition of protein synthesis, a variety of methylated derivatives of guanylic acid were prepared to test the inhibitory effects. These were synthesized by Drs. M. Ikehara and J. Fukui in the Pharmaceutical Department of Osaka University. Strong inhibitors carry a methyl group at the 7-position, suggesting that the 7-methyl group is specially important for inhibition of protein synthesis. However, all the 7-methyl guanylic acid derivatives did not show strong inhibition; 2,2,7-trimethylguanylic acid and 7,8-dimethylguanylic acid showed weak inhibition, and 2,2,7,8-tetramethylguanylic acid did not give any inhibitory effect. In these cases, the inhibitory effect of the 7-methyl group would be cancelled by steric hindrance or configurational change by other methylation around the 7-methyl group.

7-methyl guanine carries positive charge differently from other purine bases. To determine the effect of the positive charge in inhibition of protein synthesis, other similar nucleotides were prepared. Neither 1-methyladenylic acid nor 2-methylthio-7-methyl inosinic acid showed any inhibition in a wheat germ protein synthesizing system. Thus, the methylation itself at the 7-position in guanine seems to be specifically required for inhibition rather than its positive charge. The confronting nucleotide structure (cap) at the 5′-terminus of mRNA involves 2′-0-methylated nucleotide or 6-methyladenylic acid as the first nucleotide of the RNA chain. Addition of 2′-0-methylguanylic acid and 6-methyladenylic acid to the wheat germ system did not cause inhibition for protein synthesis. These results indicate that the inhibition of protein synthesis was caused by 7-methylguanylic acid residue in the confronting nucleotide structure.

It has been known that the 7-methylguanylic acid-deleted mRNA treated with tobacco pyrophosphatase loses its ability to form the initiation complex of protein synthesis. It was also shown that addition of 7-methylguanylic acid to the intact CP virus mRNA or the TMV RNA caused inhibition of the formation of the initiation complex.

The 7-methylguanylic acid-blocking structure is commonly involved in eukaryotic mRNA, whereas it is not found in prokaryotic mRNA (Annual

Rep. No. 27 (1976) p. 14). It was of interest to learn if inhibition of protein synthesis by 7-methylguanylic acid is specific for the eukaryotic system. An *in vitro* protein synthesizing system was prepared from *E. coli*. Using phage MS2 RNA and TMV RNA as mRNA, the effect of 7-methylguanylic acid was measured. There is no inhibition of protein synthesis by 7-methylguanylic acid in the *E. coli* system, regardless of whether eukaryotic or prokaryotic mRNA is used. Thus, the inhibition by 7-methylguanylic acid takes place in the step of the initiation complex formation for protein synthesis with eukaryotic ribosomes. These suggest that the m^7GMP residue in the cap of mRNA performs some specific role in eukaryotic protein synthesis, especially in its initial step.

A Novel 5′-Exonuclease which Degrades Uncapped Messenger RNA

Kunitada SHIMOTOHNO and Kin-ichiro MIURA

Our previous work using a wheat germ cell-free system showed that the 5′-terminal cap structure in eukaryotic mRNA is important in protein synthesis, especially in the formation of the initiation complex. It was also shown that the cap structure protects mRNA from nuclease attack and stabilizes mRNA. In these experiments the intact mRNA was compared with uncapped mRNA, which was prepared by treatment with pyrophosphatase from cultured tobacco cells (Annual Rep. No. 27 (1976) p. 20; Shimotohno, K., Kodama, Y., J. Hashiomoto and Miura, K., Proc. Natl. Acad. Sci. USA, **74**: 2734 (1977)). After incubation of uncapped mRNA with wheat germ extract (S–30 fraction), mRNA was degraded to nucleotides, while the intact mRNA was not. This result suggest the presence of a 5′-exonuclease acting on the uncapped mRNA in wheat germ extract. We therefore attempted to purify the enzyme and to characterize it.

The S–30 fraction (soluble fraction in centrifugation at $30{,}000\times g$) of wheat germ extract was centrifuged at $100{,}000\times g$ for 2 hrs to obtain a postribosomal fraction. The fraction containing the exonuclease activity was obtained by the following procedures: column chromatographies with DEAE-cellulose, Sepharacryl S–200 and hydroxyapatite. The enzyme activity was surveyed employing two kinds of substrate, intact mRNA and the decapped mRNA which had been treated with tobacco pyrophosphatase. The exonuclease was purified about 20-fold at the final step of hydroxyapatite chromatography.

Optimal conditions for the enzyme reaction were studied. The enzyme requires 0.2 mM Mg^{++}. The optimal temperature and pH are 25°C and pH 6.0. The enzyme hydrolyzes uncapped mRNA but cannot attack intact mRNA. This result suggests that the enzyme recognizes the 5′-terminal structure of the substrate RNA strictly. Using RNA substrates which carry various structures at the 5′-termini, the degradation behavior of RNA upon enzyme treatment was tested in detail, and it was found that the enzyme cannot attack 5′-hydroxylated RNA or the 5′-capped RNA, though mono, di- or triphosphorylated RNA's were hydrolyzed.

Degradation products produced by this enzyme were analyzed by paper electrophoresis. Only mononucleotides were detected. The mononucleotides obtained here were not converted to nucleosides by further incubation with 3′-phosphatase (*Penicillium* nuclease P), so it was concluded that the enzyme gives nucleoside 5′-monophosphates as final products from RNA.

Decapped mRNA was incubated with the enzyme for various times and the degradation of RNA was followed by centrifugation in a glycerol density gradient. It is suggested that the enzyme acts on RNA in an exonucleolytic mode. Next the direction of hydrolysis by the enzyme was studied. Decapped CPV mRNA, which was labelled with ^{32}P at the 5′-terminus and ^{3}H at the 3′-terminus, was incubated with this enzyme and the kinetics of hydrolysis were observed. Under the optimal conditions for this reaction, only radioactivity of ^{32}P was detected in the cold ethanol-soluble fraction. When the RNA was incubated with venom phosphodiesterase (Worthington Co.), the same percentages of radioactivity of ^{32}P and ^{3}H were detected in the ethanol-soluble fraction at a given time of incubation. Thus it was confirmed that the enzyme attacks RNA from the 5′-terminus in an exonucleolytic manner. This 5′-exonuclease activity is different from that of any exonuclease reported previously. This exonuclease would degrade mRNA from the 5′-terminus only after the 5′-terminal cap structure had been removed by an enzyme such as a specific pyrophosphatase found in tobacco cells.

Nucleotide Sequence around the Replication Origin of Polyoma Virus DNA

Eiichi SOEDA and Kin-ichiro MIURA

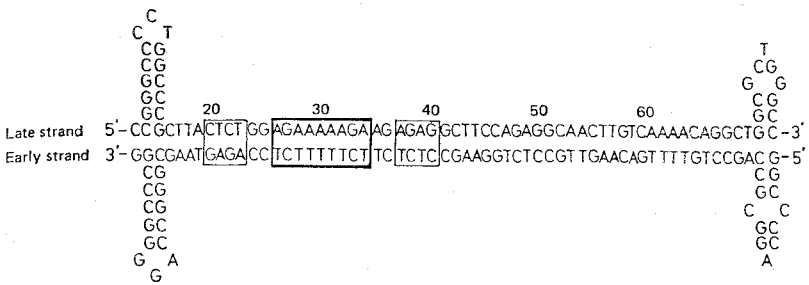
Structural studies on the DNA of small tumor viruses are proceeding

intensively as an initial step toward understanding the function of eukaryotic DNA as well as elucidating the process of tumorigenesis. Mouse polyoma virus has been well studied, as has simian virus 40 (SV 40). For SV 40, analysis of its nucleotide sequence has progressed repidly (W. Fiers, *et al.* in Belgium and S. Weissman, *et al.* in U.S.A.). It is necessary to determine the nucleotide sequence of polyoma virus DNA and compare it with that of SV 40 to clarify the common structure for function. The starting point of DNA replication has been located on both SV 40 and polyoma DNA. Further, both the transcription starting points of "early" and "late" viral mRNAs are located at regions close to the origin of DNA replication. Therefore, studies on the region around the replication origin will provide much information on the initial step in DNA replication and RNA synthesis.

The DNA replication of polyoma virus is initiated at a unique site of the viral genome, which has been mapped at 71 ± 3 map units. Some DNA fragments obtained by restriction endonucleases (HapII, HhaI and AluI) cover the origin of DNA replication. Maxam and Gilbert have developed a method for determining the nucleotide sequence in DNA by using DNA fragments labeled at the 5′ terminus with [^{32}P]phosphate by polynucleotide kinase. Because it is necessary to label the terminal part of the DNA fragment for this analysis, they labeled the 5′ terminus with ^{32}P by polynucleotide kinase. We have developed a technique for 3′-terminal labeling by the use of bacteriophage T4-induced DNA polymerase in place of polynucleotide kinase and have applied it to the polyoma DNA fragment to assess the fidelity of the analysis method with the DNA polymerase; the technique was found to be effective and convenient when compared with polynucleotide kinase. The sequences deduced from the 3′-termini of the strands of the fragment coincided almost with those derived from the 5′-terminal labeling with polynucleotide kinase. The ^{32}P-labeled fragment was split and analyzed with gel electrophoresis according to Maxam and Gilbert.

The nucleotide sequence thus derived is shown in a possible transient secondary structure as follows.

A long cluster of pyrimidines is included between 24 and 41. In this region a characteristic true palindrome sequence of T-C-T-T-T-T-T-C-T (or A-G-A-A-A-A-A-G-A) from 26 to 34 is contained. This region is sandwiched between two small symmetrical regions with a 2-fold rotational axis (20–23 and 37–40). The sequenced fragment may be characterized



also by the G+C)-rich region from 1 to 15 with a 2-fold rotational axis of symmetry, which possibly forms double hairpin loops, as well as the other (G+C)-rich region from 71 to 83.

Because mouse polyoma virus and SV 40 have similar characteristics, there is a hypothesis that the two viruses are descended from the same ancestor. If this is the case, a common structure would be conserved in a functionally important region of DNA. Comparing the polyoma nucleotide sequence analyzed here with the corresponding region in SV 40 DNA, which was sequenced by Weissman *et al.*, a characteristic symmetrical region containing a five-A stretch from 28 to 32 in the polyoma late strand fits the similar regions in SV 40 DNA. Other common long sequences which were found both in polyoma and SV 40 DNA were C-A-G-A-G-G-C, (from 46 to 52 in polyoma) and G-A-G-G-C-T-T (from 38 to 44 in polyoma), the latter is also contained in phage ϕX174 DNA near the replication origin. However, these are not symmetrical sequences.

There might be similarities among the nucleic acid structures having a common function, even among very different organisms, as shown in the cloverleaf structure of tRNA. Recently Tomizawa *et al.* showed that the replication origin of colicin El plasmid DNA contains a five-A stretch, where primer RNA switches to DNA, and both sides of this region are flanked by (G+C)-rich regions. This feature is quite similar to the structure of the polyoma DNA segment mentioned here. The A+T cluster flanked by (G+C)-rich regions has been found also at the origin of replication in phage ϕX174 DNA, although the region does not show apparent symmetry. Such a characteristic structure may be common for the starting of DNA synthesis in eukaryotes as well as in prokaryotes. Moreover, it has been shown that the promoter region contains a common

sequence rich in the A·T pairs near the initiation point of transcription. Because the cluster of the A·T base pairing must be loosened easier than the (G·C)-rich region, it is reasonable to say that the A·T cluster is involved in the starting point of DNA replication and RNA synthesis.

If the nucleotide sequence in the present segment of polyoma DNA is aligned with the corresponding region of SV 40 DNA by inserting some gaps, we can observe an extensive similarity. Fifty-eight nucleotides of the 82 (70%) of the polyoma segment are the same as SV 40. It is known that recombination occurs most frequently in this region; many variants with addition and deletion around the replication origin have been isolated so far, including viable and defective mutants. If such structural changes occurred also in the course of evolution, it is likely that polyoma virus and SV 40 have descended from a common ancestor.

This work was collaborated with Prof. Genki Kimura, Kyushu University School of Medicine. The contents were published in FEBS Lett. **79**, 383–389 (1977) and Proc. Natl. Acad. Sci. USA **75**, 162–166 (1978).

A Temperature-Sensitive Mutant of *Escherichia coli* with an Altered RNA Polymerase β' Subunit

Masahiro SUGIURA

DNA-dependent RNA polymerase of *E. coli* (EC 2.7.7.6) consists of four subunits and has the structure $\alpha_2\beta\beta'\sigma$. This enzyme plays a central role in genetic transcription in bacteria. The isolation of mutants with altered RNA polymerase is, therefore, essential to the study of transcription and its control mechanisms.

We have isolated several temperature-sensitive (ts) RNA polymerase mutants by the procedure described previously (this Report, No. 27, pp. 22–24, 1976). One strain called JE10092 was studied in detail. The P1 transduction experiments showed that the ts cell growth was due to the ts RNA polymerase and that the map order was *argH—rif*(β)*—rpoC92* (the ts mutation in JE10092). To identify the defective subunit in the ts enzyme, the subunit reconstitution experiment was carried out by mixing excess amount of a wild-type subunit with the ts enzyme. Only the ts RNA polymerase mixed with wild-type β' subunit resulted in temperature-resistant. This indicated that the ts mutation (*rpoC92*) was located in the gene for β' subunit of the RNA polymerase.

The RNA polymerase of JE10092 was easy to purify and was as stable as the parent (PA3092) enzyme up to at least 6 months. The most prominent characteristic of this enzyme was its reversible temperature sensitivity in *in vitro* reaction systems. When a reaction mixture containing the ts enzyme was incubated for 10 min at 43° and then transferred to 30°C, the activity was restored. No recovery was, however, observed when rifampicin was added simultaneously with the temperature sift-down. This indicated that high temperature affected the initiation of RNA synthesis. The ratios of activity at 43°C to 30°C differed significantly with templates used: T4-DNA, 0.19, calf thymus-DNA, 0.31, T7-DNA, 0.34, λ-DNA, 0.48, ϕX174 RF-DNA, 0.65. The results suggested that the β' subunit played an important role in promotor selection.

This work was done in collaboration with K. Segawa, K. Yoshinaga, F. Yu, N. Ito, S. Yasuda and Y. Hirota, and was published in Biochem. Biophys. Res. Commu. **76**, 739–745 (1977) and was presented at the 11th FEBS Meeting, Copenhagen, August 1977.

Physiological Studies on a Temperature-Sensitive *Escherichia coli* Mutant with an Altered RNA Polymerase β'-Subunit

Masahiro SUGIURA

Some *in vivo* properties of an *E. coli* temperature-sensitive (ts) mutant (JE10092) with an altered RNA polymerase β'-subunit were studied.

The mutant cells grew in L-broth at 30°C with a doubling time of 90 min. When the log-phase culture was shifted to 43°C, the turbidity stopped to increase within 1 hr and the colony-forming ability was reduced exponentially after an initial transient increase. The optimal growth temperature was 32°C.

When the mutant culture was shifted from 30°C to higher temperature, the (^{3}H)uridine incorporation decreased immediately to 85% at 37°C, 55% at 40°C and 6% at 43°C as compared with that at 30°C. The observed drop in (^{3}H)uridine at high temperature was due to the reduction of the rate of RNA synthesis which was examined by a pulse-labeling method. RNA synthesis was recovered immediately upon transfer to 30°C. DNA synthesis, protein synthesis and nucleoside triphosphate formation continued after the temperature shift-up.

The most prominent characteristic of this mutant was the immediate

cessation of RNA synthesis after the shift to the nonpermissive temperature. Many ts RNA polymerase mutants so far reported did not show such an immediate block of RNA synthesis at high temperature. Therefore, our mutant JE10092 is more useful for studies on the subunit function and the overall control of transcription within the cell.

This work was done in collaboration with K. Yoshinaga and was published in Biochem. Biophys. Acta **479**, 172–179 (1977).

Joining of Ribooligonucleotides with T4 RNA Ligase and Identification of the Oligonucleotide-Adenylate Intermediate

Masahiro SUGIURA

RNA ligase has been found in T4-infected *E. coli* cells. Originally, the ligase activity was found to promote the cyclization of ribopolynucleotides of chain length around 40 particularly well and a minimum chain length for cyclization was reported as being eight. Shorter oligonucleotides were joined using an excess of the 3′-hydroxyl component.

T4 RNA ligase was purified from T4 amN82×E1140-infected *E. coli* A19 (RNase I$^-$), which was low in RNase activity. The RNA ligase was found to join A-A-A-A-A-A and ^{32}pU-U-U-U. In this reaction the pyrophosphate of *pU-U-U-U and pA was isolated by chromatography on a RPC-5 column, besides the joined product and the starting materials. This pyrophosphate was shown to be an intermediate in the joining reaction because of the fact that coupling with A-A-A-A-A-A to give the decanucleotide could be performed in the absence of ATP. The structure of the oligonucleotide-adenylate was determined by enzymatic digestion with base-nonspecific nuclease and venom phosphodiesterase. Further evidence for the proposed structure was obtained by isolation of the intermediate obtained by using *pU-U-U-U and [α-^{32}P]ATP. This pyrophosphate gave *pA and *pU by treatment with venom phosphodiesterase. The ligation reaction, therefore proceeds as follows,

$$\mathrm{ATP} + {}^{*}\mathrm{pU\text{-}U\text{-}U\text{-}U} \rightarrow \mathrm{O}\begin{matrix}\diagup \mathrm{pA} \\ \diagdown {}^{*}\mathrm{pU\text{-}U\text{-}U\text{-}U}\end{matrix} + \mathrm{PPi}$$

$$\mathrm{A\text{-}A\text{-}A\text{-}A\text{-}A\text{-}A} + \mathrm{O}\begin{matrix}\diagup \mathrm{pA} \\ \diagdown {}^{*}\mathrm{pU\text{-}U\text{-}U\text{-}U}\end{matrix} \rightarrow \mathrm{A\text{-}A\text{-}A\text{-}A\text{-}A\text{-}A}{}^{*}\mathrm{pU\text{-}U\text{-}U\text{-}U} + \mathrm{AMP}$$

This work was done in collaboration with E. Ohtsuka, S. Nishikawa and M. Ikehara, and was published in Nucleic Acids Res. **3**, 1613–1623 (1966).

Joining of Synthetic Ribotrinucleotides with Defined Sequences Catalyzed by T4 RNA Ligase

Masahiro SUGIURA

T4 RNA ligase has been shown to catalyze intra- and intermolecular joining of the 5′-phosphate and the 3′-hydroxyl group of oligo and polynucleotides using ATP as cofactor.

We found that RNA ligase catalyzed the joining of pC-C-Ap with C-A-A in the synthesis of C-A-A-C-C-Ap, which has the sequence of the *Escherichia coli* $tRNA_f^{Met}$ 3′-end. pC-C-A to various synthetic ribotriplets, such as C-C-A, A-A-A, C-C-C, U-U-U, U-A-G, C-C-G and U-U-C, was performed as well as joining to the partially substituted trimers with a photolabile *o*-nitrobenzyl group, C-Anbzl-A and C-C-Anbzl. The yields were C-A-A-C-C-A (69%), C-C-A-C-C-A (38%), A-A-A-C-C-A (66%), C-C-C-C-C-A (71%), U-U-U-C-C-A (50%), U-A-G-C-C-A (23%), C-C-G-C-C-A (43%) and U-U-C-C-C-A (46%). C-Anbzl-A was a slightly poorer acceptor than C-A-A and C-C-Anbzl did not serve as an acceptor. Recongnition of acceptor molecules by RNA ligase is discussed in terms of affinity of ligonucleotides for the enzyme.

This work was done in collaboration with E. OHTSUKA, S. NISHIKAWA, R. FUKUMOTO, S. TANAKA, A. F. MARKHAM and M. IKEHARA, and was published in Eur. J. Biochem. **81**, 285–291 (1977).

Molecular Cloning of Tobacco Chloroplast Ribosomal RNA Genes

Masahiro SUGIURA and Jun KUSUDA

Chloroplasts of higher plants contain ribosomes of similar sizes to those of prokaryotes. Chloroplast DNA exists as a circular molecule with a molecular weight of around 10^8 daltons and codes for its rRNA's. In order to analyze the fine structure and the expression of chloroplast rRNA genes, we have constructed recombinant molecules between *E. coli* plasmid pMB9 and chloroplast rRNA genes from tobacco leaves.

Chloroplast DNA was purified from healthy mature tobacco leaves by using differential and sucrose gradient centrifugations. The chloroplast

DNA was digested with restriction endonuclease EcoRI. The DNA fragments were fractionated by 1% agarose gel electrophoresis and transferred to a Millipore filter according to Southern. Chloroplast 23S and 16S rRNA were paritially hydrolyzed by alkali and then labeled with ^{32}P at their 5′-ends by using T4 polynucleotide kinase and (γ-^{32}P)ATP. ^{32}P-rRNA probe was hybridized to the filter-bound DNA fragments. The resulting autoradiograph indicated that the chloroplast rRNA hybridized to the fragments of molecular weights of 1.9×10^6 and 2.8×10^6 daltons.

Total EcoRI fragments of the chloroplast DNA and pMB9 digested with EcoRI followed by alkaline phosphatase treatment were joined using T4 DNA ligase, and the ligated DNA was used to transform *E. coli* HB101. 373 tetracycline-resistant transformants were screened for the presence of DNA complementary to (5′-^{32}P)rRNA using the colony hybridization procedure developed by Grunstein and Hogness. The 14 clones hybridized to the ^{32}P-rRNA. The recombinant plasmids containing a 1.9×10^6 fragment (TC001) and a 2.8×10^6 fragment (TC309) were selected by agarose gel electrophoresis followed by filter hybridization.

II. MICROBIAL GENETICS

Involvement of Penicillin Binding Protein in Cross-Linking of Murein

Hideho SUZUKI, Yukinobu NISHIMURA and Yukinori HIROTA

In order to elucidate the role of penicillin binding protein (PBP) in murein biosynthesis, a series of multiple mutants having various combination of mutations in PBPs were constructed. Particulate fractions were prepared from them and activities for murein synthesis were investigated by N-acetylmuramylpentapeptide-dependent incorporation of N-acetylglucosamine with UDP-linked precursors. The level of murein synthesis *in vitro* was essentially unchanged in the mutants defective in PBP-1a(*ponA*), PBP-2(*rodA*) or PBP-3(*ftsI*). In contrast, practically no murein synthesis *in vitro* was found in two independent mutants for PBP-1b(*ponB704* and *ponB1085*), although lipid intermediates were synthesized at a level comparable to that in the wild type. In another PBP-1b mutant(*ponB353*) the particulate fraction incorporated the precursor into murein at a lower level (about 60% of the wild type). These different characteristics are specific for each mutation while the general phenotype of these strains is the lack of PBP-1b. The *in vitro* products were analyzed for cross-linking by lysozyme digestion and subsequent separation of muropeptides by gel-filtration. The ratio of bis(disaccharide-peptides) to disaccharide-peptides was about 0.15 in the *ponB353* mutant, whereas it was 1.1 ± 0.2 in the wild type and in the mutants defective in PBP-1a, PBP-2 and PBP-3. The sharp decrease in the degree of cross-linking in the *ponB353* mutant indicates that the PBP-1b is involved in the reaction of murein cross-linking. The level of the incorporation of substrate in the *ponB353* was high enough to allow the interpretation that the extention of glycan-chain proceeded in the absence of transpeptidation and to preclude the argument that the failure in cross-linking might result from the absence of extended glycan-chains that could serve as substrates for transpeptidation.

The degree of cross-linking *in vivo* was examined in *dap*$^-$ *lys*$^-$ auxotrophs of mutants defective in PBPs by following the incorporation of ^{14}C-diaminopimelic acid into lysozyme digests. In contrast to the *in vitro* experiments, the degree of cross-linking *in vivo* in all three of the *ponB* mutants was almost the same as that in the wild type. A double mutant

carrying *ponA*^{ts} *ponB* was shown to be temperature sensitive with respect to growth, although the parental strains carrying a single mutation, either *ponA*^{ts} or *ponB*, were not. This suggested that the loss of function of PBP-1b(*ponB*) *in vivo* was bypassed by the product of the *ponA*^{ts} gene, PBP-1a^{ts}. Furthermore, the double mutation, *ponA*^{ts} *ponB*, did not decrease the degree of cross-link in the murein synthesized *in vivo* at 42°, in spite of causing cell lysis. In the presence of benzylpenicillin or cephaloridine at a dose leading to cell lysis, synthesized murein still showed the normal degree of cross-link, although gross murein synthesis was decreased to less than one third of the control in one hour. To explain these results, the following working hypothesis was proposed.

There must be at least two reactions for cross-linking *in vivo*: one is comparatively insensitive to β-lactam antibiotics and another is sensitive to β-lactam antibiotics. Transpeptidation which is required for the lateral extention of the sacculus is supported by the former reaction which constitutes a major part of cross-linking reactions for cell elongation. PBP-1b is involved in the latter reaction which is a primary target of β-lactam action. The PBP-1b specific reaction is functional at a certain stage of the cell cycle and arrest of this reaction, either by action of β-lactams or as a result of a genetic defect, would bring about a cleft on succulus leading to bulge formation and cell lysis. Extrusion of cytoplasm out of the cleft may diminish or discontinue overall murein synthesis as a result of disorganization of structural contact of cytoplasmic membrane and murein sacculus. Bulge formation appears to be localized at a specific site which has a capacity to differentiate into a septum. Thus, the PBP-1b may function to cross-link muropeptides at the potential division site developed at a certain stage of the cell cycle. The PBP-3 may act to direct the differentiation of this site into a septum, alternatively these two PBPs may cooperate in the reaction leading to septation. Evidence for such cooperation comes from studies of a multiple mutatnt defective in both PBP-1b and PBP-3. This mutant was found to be osmotically sensitive, requiring a hypertonic medium for proper progagation. Transfer of the mutant to ordinary nutrient broth caused progressive cell lysis. This result suggests the possible requirement of cooperation of PBP-3 in murein synthesis at some specific stage of a cell cycle.

Control of Chromosome Replication by an Integrated ColEl Plasmid in *Escherichia coli*

Seiichi YASUDA, Yukinobu NISHIMURA and Yukinori HIROTA

The ColEl plasmid is known as a unique replicon which has different characteristics from those of other replicons, such as E. coli chromosome, F and R plasmids. There are about 20 copies of ColEl plasmid DNA per cell. Initiation of replication is not inhibited by chloramphenicol, and elongation is catalyzed by DNA polymerase I. Integration of ColEl plasmid into the chromosome of *E. coli* K12 has not been reported, and no integrative suppression by ColEl has been observed for a thermosensitive DNA initiation mutation, *danA*^{ts}.

We report here an integrative suppression of *dnaA*^{ts} mutation by a synthetic ColE$_1$ plasmid. When a plasmid, pLC26-3, carrying the *ilv* region of the *E. coli* chromosome (Clarke, L. and J. Carbon 1976. Cell **9**: 91) was introduced into a *dnaA*^{ts} mutant, CRT46, thermoresistant revertants were obtained at a frequency of about 4×10^{-5}. The reversion frequency was about 50-fold higher than that of CRT46 which did not carry the plasmid. These revertants grew slowly at 42°C. This higher frequency of appearance of thermoresistant reversion in the CRT46 which carries a ColEl plasmid could be interpreted as an integrative suppression (Nishimura, Y., L. Caro, C. M. Berg and Y. Hirota 1971. J. Mol. Biol. **55**: 441) by the ColEl plasmid. A homology in nucleotide sequences between the *E. coli* chromosomal fragment in the plasmid and the corresponding region of host chromosome could provide the recombination site. This would result in the formation of a fused replicon in which the ColEl plasmid is integrated into the host chromosome. Such bacteria could replicate their DNA using the replication machinery of the integrated CoEl replicon.

Immunity to colicin El in these revertants, a phenotype of *E. coli* carrying ColEl genone, was co-transducible with the *met* gene, which is located near the *ilv* gene, and the frequency of joint transduction of the two outside markers was lowered. These results indicated that the synthetic ColEl plasmid was integrated into *ilv* region and that the region was stretched to the corresponding length of the plasmid. The profile of CsCl-ethidium bromide density centrifugation of DNA extracted from the revertants showed the absence of a satellite band which could be responsible for the plasmid DNA in cytoplasmic state. This result confirmed that the plasmid DNA in the revertants was in an integrated state.

III. BIOCHEMICAL GENETICS AND IMMUNOGENETICS

Estimation of Genetic Distance between the Japanese and the European Wild Mice, *M.m.molossinus* and *M.m.domesticus*

Kazuo MORIWAKI and Mitsuru MINEZAWA

Recently several investigators have reported circumstantial evidences suggesting rather remote genetic distance between the Japanese wild mouse, *Mus musculus molossinus*, and the European one, *M.m.domesticus*, from which most of the laboratory mice likely originated. Those are the differences in gamma-globulin heavy chain genes (Liberman and Potter 1969, J. Exp. Med. **130**: 519), Hbb alleles (Nishimura *et al.* 1973, Exp. Animals **22**: 187), satellite DNA contents (Rice & Straus 1973, P.N.A.S. **70**: 3546), chromosome C band patterns (Dev *et al.* 1975, Chromosoma **53**: 335) and Isocitrate dehydrogenase-1 alleles (Minezawa *et al.* 1976, This report **26**: 23) and also abnormal meiotic figures in F_1 hybrid between the two subspecies (Roderick 1971, Mut. Res. **11**: 59).

We attempted to estimate their genetic distance quantitatively using the data on the allelic frequencies of various biochemical characters reported previously by us (Minezawa *et al.* 1976, This report **26**: 23) and other workers (Selander *et al.* 1969, Evolution **23**: 379; Selander & Yang 1969, Genetics **63**: 653; Staats 1976, Cancer Res. **36**: 4333). They are summarized in Table 1. Allelic frequencies of those 10 loci, 6 polymorphic and 4 monomorphic (albumin, transferrin, erythrocytic esterase and prealbumin), were applied to Nei's equation (1975 Molecular population genetics and evolution, North Holland) for calculating standard genetic distance (D).

$$D = -\log_e J_{XY}/\sqrt{J_X J_Y}$$

Calculated D is 0.041 between *M.m.domesticus* in Europe and that in North America, 0.327 between *M.m.domesticus* and *M.m.musculus* and 0.514 between *M.m.domesticus* and *M.m.molossinus*. Nei (1975) estimated D valuse between *M.m.musculus* and *M.m.domesticus* as 0.196 using 41 loci reported by Selander *et al.* (1969). If we use more loci for the calculation of D between *M.m.molossinus* and *M.m.domesticus*, it should be reduced to a considerable extent. Moreover, considering 'deme' structure characteristic in wild mice, estimated D value could be around 0.3. Ac-

Table 1. Allelic frequencies of six protein loci in the three subspecies of mouse, *Mus musculus*

Locus	Allele	Name of subspecies and allelic frequencies				
		M.m. molossinus in Japan	*M.m. musculus* in Europe	*M.m. domesticus* in Europe	*M.m. domesticus* in U.S.A.	Laboratory mice
Esterase-1 (Es-1)	a	.74	.99	.00	.01	.16
	b	.18	.01	1.00	.98	.84
	c	.07	.00	.00	.01	.00
Esterase-2 (Es-2)	a	.09	.00	.00	.09	.08
	b	.03	.07	1.00	.84	.90
	c	.88	.93	.00	.01	.02
	d	.00	.00	.00	.04	.00
Esterase-3 (Es-3)	a	1.00	.00	.00	.00	.33
	b	.00	.44	.35	.75	.05
	c	.00	.56	.65	.23	.62
	d	.00	.00	.00	.01	.00
Esterase-5 (Es-5)	a	.91	.95	.74	.85	.06
	b	.01	.05	.26	.15	.94
	c	.08	.00	.00	.00	.00
Isocitrate dehydrogenase (Id-1)	a	.08	.12	.93	.55	.58
	b	.78	.88	.07	.46	.42
	c	.13	.00	.00	.00	.06
	d	.01	.00	.00	.00	.00
Hemoglobin beta-chain (Hbb)	d	.40	.33	.16	.20	.53
	s	.08	.67	.84	.80	.46
	p	.60	.00	.00	.00	.01
Number of samples		194	69	30	1221	40
References		Minezawa *et al.*, 1977	Selander *et al.*, 1969	Selander *et al.*, 1969	Selander & Yang, 1969	Staats, 1976

cording to Nei (1975), divergence time is obtained by 5×10^6D. $D=0.3$ gives approximately 1.5×10^6 years. This value well reconcile with another estimation (Benveniste *et al.* 1977, J. Virol. **21**: 849). Allelic frequency data in Table 1 also strongly suggest that most of the laboratory mouse originated from the European wild mouse, *M.m.domesticus.*

Distribution of Three H-2 Antigenic Specificities in Various Species of Wild Rodents

Kazuo MORIWAKI and Toshihiko SHIROISHI

Distribution of three H-2 antigenic specificities, KD5, K8 and D13, in various species of wild rodents was surveyed by a quantitative abbsorption of monospecific alloantisera with either erythrocytes or spleen lymphocytes of the animals to be examined. Results are summarized in Table 1. The alloantisera for detecting KD5, K8 and D13 were given by NIH, designations of which are D-5bAF, D-8 and D-13 respectively. Residual activity of each antiserum after the absorption was assayed by cytotoxicity test using ^{51}Cr labelled lymphocytes as targets obtained from either B10 or B10.D2 congenic mice. Percent residual activity is expressed as follows:

$$\frac{\text{Released } ^{51}\text{Cr cpm by absorbed antiserum} - \text{blank}}{\text{Released } ^{51}\text{Cr cpm by unabsorbed antiserum} - \text{blank}} \times 100$$

In Table 1, +, ± and − correspond to three ranges of the average percentage of 2 or 3 individuals, 0–20%, 20%–50% and 50%–100%. Within *Mus musculus* species, those H-2 antigenic specificities were commonly observed. Previous studies have also demonstrated that H-2.5 specificity widely distributes both in *M.m.domesticus* (Europe) and *M.m. molossinus* (Japan) (Mickova & Ivanyi 1976, Folia Biol. **22**: 169; Moriwaki *et al.* 1976, This report **26**: 20). Out of *M.musculus* species, H-2.5 could not be detected as far as the present survey is concerned, while H-2.8 has widely been observed within subfamily murinae. Though serological survey of common antigens in the wild rodents often involves complex methodological problems such as crossreactivity of antibody, it should be of great interest that the commonest specificity in *Mus musculus* species does not exist in many other rodent species where the other H-2 specificity not being very frequent in *Mus species* widely distributes.

Table 1. Distribution of H-2 antigens in various rodents

Family	Subfamily	Genus and species	H-2 antigenic specificities					
			KD-5		K-8		D-13	
			E*	L*	E	L	E	L
Cricetidae	Gerbillinae	*Meriones unguiculatus*	−	−	−	−	−	−
	Microtinae	*Clethrionomys bedfordiae*	−	−	−	−	−	−
		Microtus montebelli	−	−	−	−	−	−
Muridae	Murinae	*Millardia meltada*	−	−	−	+	−	±
		Apodemus speciosus	−	−	−	±	−	−
		Rattus rattus	−	−	−	+	−	−
		Rattus flavipectus	−	−	−	+	−	−
		Rattus norvegicus	−	−	−	+	−	−
		Mus platythrix	−	−	−	+	−	+
		Mus caroli	−	−	−	−	−	−
		Mus musculus molossinus	+	+	±	+	±	+
		Mus musculus castaneus	+	+	−	±	−	+
		Mus musculus domesticus	+	+	+	+	+	+
		Mus musculus bacterianus	+	+	+	+	−	+

* E: erythrocytes. L: spleen lymphocytes.

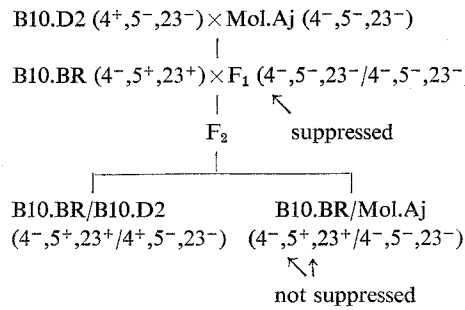
Genetic Regulation for the Expression of Erythrocytic H-2 Antigens in a Mouse Intersubspecies Hybrid

Kazuo MORIWAKI and Toshihiko SHIROISHI

So far mouse H-2 genes are to be expressed codominantly in most adult tissues. However, in F_1 hybrid between two mouse subspecies, *Mus musculus domesticus* (European wild mouse and most laboratory mouse) and *M.m. molossinus* (Japanese wild mouse), expression of erythrocytic H-2 antigens introduced from *domesticus* is frequently suppressed as previously described by us (Moriwaki & Shiroishi 1976, This report **27**: 36). This suppression behaved like a dominant trait in F_1 hybrid. We examined further the mode of H-2 expression in F_2 progenies.

Firstly, a B10.D2 strain female was mated with a Mol.Aj (*M.m. molossinus*) male to obtain F_1 hybrids. Erythrocytic H-2 antigen specificities detected are 4^+, 5^- and 23^- in the former, 4^-, 5^- and 23^- in the latter, and 4^-, 5^- and 23^- in the F_1 hybrids. Thus H-2.4 from B10.D2 was clearly suppressed on the erythrocytes of the F_1 hybrids as already stated, whereas it was distinctly detected on their spleen lymphocytes.

Secondly, the F_1 male was mated with a B10.BR female to produce F_2 progenies. H-2 antigenic specificities of B10.BR mouse are 4^-, 5^+ and 23^+. In F_2 progenies, about half should have heterozygous H-2 chromosomes such as B10.BR/B10.D2 and the other half B10.BR/Mol.Aj.



If H-2 chromosome of Mol.Aj has some dominant suppressing factor, about 50% of F_2 progenies can not express H-2.5 and H-2.23 specificities on the erythrocytes. But our data on 16 F_2 progenies demonstrated that all of them definitely expressed H-2.5 and 23 specificities on the erythrocytes, though H-2.4 positive individuals were 7 out of 16.

This result excludes the possibility that a single dominant gene is governing suppression mechanism of H-2 antigens on erythrocytes in this case.

Developmental Changes of H-2 Antigens on Postnatal Mouse Erythrocytes

Toshihiko SHIROISH and Kazuo MORIWAKI

Pizarro *et al.* already reported temporal patterns of development of H-2 antigens on erythrocytes (Pizarro *et al.* 1961). In their study, it was difficult to trace the quantitative developmental changes of H-2 antigens exactly, because all their observation were conducted by a hemagglutination method.

In the present work, we performed the quantitative absorption of anti-H-2 sera, and subsequently assayed the residual activity by the cytotoxicity method using ^{51}Cr labelled lymphocytes. We examined KD3, D4, K5, K23 specificities of H-2 antigens on postnatal B10.A ($H\text{-}2^a$) mouse erythrocytes. Results are summarized in Table 1. The quantity of antigens is expressed in terms of absorbing capacity, which means % of absorbed cytotoxic activity against the full cytotoxic activity of non-absorbed allo-antisera.

Table 1. Temporal patterns of development of H-2 antigens on postnatal B10.A erythrocytes

H-2 antigenic specificities examined	absorbing capacity (%) in each stage*				
	1 day	3 days	8 days	13 days	8 weeks
KD3	11	45	37	66	76
D4	18	33	44	65	90
K5	23	11	53	85	100
K23	12	53	42	50	72

* Each stages are designated days after birth.

From these results, we found out that even new born mouse (1 day) already expressed each H-2 antigens on erythrocytes at 10–20% of adult mouse, generally they increased untill 8 weeks. It is, however, notable that there are some difference of developmental patterns among each H-2 antigenic specificities examined in this experiment. For example, H-2D.4 increased lineally from 1 day to 8 weeks, while H-2KD.3 and H-2K.23

decreased once from 3 days to 8 days and after 8 days increased again untill 8 weeks. A similar pattern was observed in the development of H-2K.5 antigen, which decreased from 1 day to 3 days. The most striking findings are that these quantitative dissmilarity with respect to their developmental changes is observed within the H-2 antigens which have been believed to exist in the same H-2 molecule. Both H-2K.5 and H-2K.23 antigens are supposed to be composed on the same molecule H-2K, neverthless the patterns of development of these two antigens are different each other.

These findings are not quite agreement with Pizarro's observation. They might reflect the possible existence of independent genes which direct each H-2 antigens. But now we do not have any solid explanation for this phenomenon.

Allelic Constitution of *Hbb* Locus in Asian House Mouse Subspecies

Mitsuru MINEZAWA[1] and Kazuo MORIWAKI

One of the Asian mouse subspecies, *Mus musculus molossinus* (habitate in Japan), has rather different genetic constitution from the Western hemisphare wild mouse (Minezawa *et al.* 1976, This Report **26**: 23–25). Its *Hbb* (hemoglobin beta-chain) locus consisted of mainly Hbb^p and Hbb^d alleles. But Hbb^s allele was rare and considered to be introduced to Japanese wild population very recently, though it is common as well as Hbb^d in the Western hemisphare (Minezawa *et al.* 1977, This Report **27**: 33–34). It is not yet clear whether the frequent occurrence of Hbb^p allele is unique to the Japanese population or general feature in the Asian mice. This would be crucial ploblem to reveal the evolutionary process of *Hbb* gene of *Mus musculus* and differentiation of *Mus musculus* subspecies.

Twenty three wild mice were collected from four Asian countries to examine their *Hbb* types. Twelve were obtained from Philippines by courtesy of Dr. J. S. Masangkay, 11 from Quezon and 1 from Batangas; Six from Taichung in Taiwan by courtesy of Dr. T. Y. Ku; Three from Lahore in Pakistan and two from Kabul in Afghanistan by courtesty of Mr. H. Ikeda. Two fancy mice from Bangkok in Thailand were also examined. The results are summarized in Table 1. Allelic constitution similar to Japanese population was observed and the frequencies of Hbb^p and Hbb^d fluctuated depending on the locality of collection. Putting to-

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Table 1. Phenotypes of hemoglobin beta-chain (*Hbb*) of house mouse from five Asian countries

Country	No. of Mice	No. of Phenotype		
		P	PD	D
Taiwan (wild mouse)	6	5	1	
Philippines (wild mouse)	12	2		10
Pakistan (wild mouse)	3	2		1
Afganistan (wild mouse)	2	1	1	
Thailand (fancy mouse)	2	2		

gether the other observations (Gilman 1974, Ann. N.Y. Acad. Sci. **241**: 416–433; Morton and Tobin 1977, Biochem. Genet. **15**: 101–108), *Hbb* locus of wild mouse population in the Eastern Asia generally consisted of Hbb^d and Hbb^p alleles, and Hbb^s alleles is rare or inexistent. This geographical distribution profile of *Hbb* alleles, especially inexsistence of Hbb^s allele, is inconsistent with Gilman's hypothesis, which supposed coexistence of two ancestral alleles, duplet which consist of two close linked genes, and Hbb^s-like singlet alleles. We could not find the cause of disappearence of singlet allele from Eastern Asia. Another possible hypothesis is that ancestral Hbb^s-like singlet allele was produced by deletion or suppression of one component of the duplet genes. On the basis of the distribution of two alleles, Hbb^p and Hbb^s, *Mus musculus* suspecies would be classified into two subspecies groups, such as Asian subspecies with Hbb^p and Hbb^d alleles and Western hemisphare subspecies with Hbb^s and Hbb^d alleles. This classification is similar to that by Marshall who proposed two major subspecies groups, *castaneus* group habitated in Eastern Asia and *musculus* group habitated in Western hemisphare and Western Asia (1977 Bull. Am. Mus. Nat. Hist. **158**: 172–220). The disagreement between Marshall and we is found in Afghanistan and Pakistan mice which were classified into *musculus* group and possessed Hbb^p allele. This inconsistency is, however, well accounted by the fact that *M. m. bactrianus* inhabitated in those area revealed many mixed characters of European and East Asian subspecies, and intermingling process between two subspecies groups is now in progress in those area.

Geographical Distribution of Isocitrate Dehydrogenase-1^c in the Japanese Wild Mouse, *Mus musculus molossinus*

Mitsuru MINEZAWA[1], Kazuo MORIWAKI and Kyoji KONDO[2]

We have preliminarily reported four alleles of isocitrate dehydrogenase (*Id-1*$^{a,b,c \text{ and } d}$) locus in the Japanese wild mouse (*Mus musclus molossinus*) population. Among them, *Id-1*^{a} and *Id-1*^{b} alleles were recorded from several Western hemisphare pouplations and laboratory strains, whereas *Id-1*^{c} and *Id-1*^{d} alleles were not except for rare case (probably *Id-1*^{c}) in Hawaii (Minezawa *et al.* 1976, This Report No. **26**: 23–25). As *Id-1*^{c} allele was observed in another Asian subspecies *Mus musculus castaneus* from Thailand and Philippines, we considered this allele is specific to Asian mice.

Table 1. Allelic frequency of isocitrate dehydrogenase (*Id-1*) of wild house mouse in 14 geographical regions of Japan

Regions	No. of Mice	Allelic Freq. (%)			
		a	b	c	d
Hokkaido	19		97.4		2.6
Mainland (Honshu)					
Tohoku	14		100.0		
Hokuriku	6		100.0		
Kanto	14		100.0		
Tokai	17		97.1		2.9
Kinki	10		100.0		
San-yo	15		100.0		
San-in	13	3.8	96.2		
Shikoku	7		100.0		
Tsushima	11	27.3	13.6	59.1	
Kyushu	42	17.8	51.2	31.0	
Oosumi Isls.	3			100.0	
Amami Isls.	8	18.8	75.0	6.2	
Okinawa	1		50.0	50.0	

Geographical distribution of those *Id-1* alleles in the Japanese wild mouse population was shown in Table 1, based on 181 mice collected from 7 islands (Hokkaido, Shikoku, Tsushima, Kyushu, Oosumi Isls., Amami Isls. and Okinawa) and 7 regions of Mainland (Tohoku, Hokuriku, Kanto,

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Tokai, Kinki, San-yo and San-in). As a whole, *Id-1*^{b} was the commonest in Japan. The frequencies of four alleles, *Id-1*^{b}, *Id-1*^{c}, *Id-1*^{a} and *Id-1*^{d}, were 79.4%, 13.1%, 6.9% and 0.6%, respectively. In northern part of Japan such as Hokkaido, Shikoku and Mainland except for San-in, *Id-1* locus consists of *Id-1*^{b} allele except for two cases with *Id-1* BD phenotype. Whereas, in southern part of Japan, this locus mostly consist of two or three alleles, namely assortment of *Id-1*^{c}, *Id-1*^{b} and *Id-1*^{a}. All three mice collected from Oosumi Isls. showed *Id-1*^{c} allele in homozygous state.

Those findings may suggest a possibility that an original allele of the Japanese wild mouse population was *Id-1*^{b}. Considering its geographical distribution and electrophoretical mobility, *Id-1*^{d} allele seems to be produced from *Id-1*^{b} by one or two amino acid substitution. On the otehr hand, existence of *Id-1*^{c} allele in either Asian continent and southern islands of Japan, suggested that *Id-1*^{c} allele was immigrated into Japan from Asian continent through two routes, Korea to Tsushima route and Taiwan to Southeast archipelago route.

Electrophoretic Survey for Regional Differences of Blood Proteins in Ryukyu Mice from the Southeast Asia

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Recently Marshall (Bull. Am. Jus. Nat. Hist. **158**: 172, 1977) has classified *Mus formosanus* Kuroda, which habitats only in Taiwan and is characterized by a sharply bicolored tail, into *Mus caroli* (Ryukyu mouse) based on the morphological characters. As the measurements of external characters, head and body length and tail length, of *Mus caroli* obtained from three regions in the Southeast Asia, Okinawa, Taiwan and Thailand, revealed rather large differences, we further examined several biochemical characteristics to clarify their intraspecies relationship. Total nineteen individuals were obtained from the three regions, 5 from Naha city in Okinawa (by courtesy of Dr. Kyoji Kondo), 6 from Taichung city in Taiwan and 8 from

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4) Plant Protection Center, Taiwan
5) Cancer Institute

Lombri province in Thailand. Hemoglobin, serum esterases and serum proteins were examined by starch- and acrylamide gel electrophoresis.

Hemoglobin: *Mus caroli* possess a unique hemoglobin beta chain among *Mus* species (Gilman 1974, Ann. N.Y. Acad. Sci. **241**: 416). All mice from 3 regions exhibited this species specific hemoglobin on their electrophretic patterns.

Serum esterases: Major serum esterase in the mice obtained from Taichung showed a zymogram different from those from the other regions.

Serum proteins: Albumine and transferrin of the mice from the three regions showed almost the same pattern. Whereas several minor serum proteins observed in Taichung region were somewhat different electrophoretically from those in the other two regions.

Those results suggest a possibility that *Mus caroli* in Taiwan is genetically remote to some extent from the other two *Mus caroli* in Okinawa and Thailand, not in parallel with their geographical relationship.

Comparative Analyses of Japanese Wood Mice between Oki Islands and Mainland from the View points of Biochemistry and Cytogenetics

Hirohisa HIRAI, Kazuo MORIWAKI and Teru Aki UCHIDA[1)]

At present the wood mouse of Hondo (Japanese mainland) is classified into *Apodemus speciosus speciosus* and that of Dozen and Dogo in Oki islands *A.s.navigator*. Hiraiwa *et al.* (1958, Sci. Bull. Fac. Agr. Kushu Univ. **16**: 547) have described that the tail ratio of *navigator* is lower than that of *speciosus* and the dead center represented by the distance between the first upper molars of skull in specimens from Oki Is., especially from Dozen, is longer than that of Hondo. Judging from this, they claimed that the animals from Dozen can be distinguished from *navigator* of Dogo at a subspecies level. We attempted to dissolve this problem from the genetical viewpoints.

As a preliminary survey, we compared *A.s.navigator* from Dozen and Dogo with *A.s.speciosus* from Fukuoka and Mishima by using electrophoretic, immunological and cytogenetical techniques. Albumin, transferrin, hemoglobin, erythrocytic esterase, serum esterase-1 and -2 were analysed by the thin layer acrylamide gel electrophoresis. Fifteen per cent acrylamide

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separation gel dissolved in Tris-Hcl buffer (pH 8.9) was prepared in 1 mm thick between two glass plates of 170×180×3 mm each. Spacer-sample gel with 3.2% acrylamide in Tris-Hcl buffer (pH 6.7) was put on that. Thirteen samples were analysed at once usually by 4 hours run at 400 V constant voltage under refrigeration. Coomasie brilliant blue was used for staining protein on the gel. Esterase bands were detected by the incubation of gels at 37°C for 20 minutes in a mixture (pH 6.8) of 0.03% naphthyl acetate and 0.1% fast blue RR salt.

Though there was no detectable difference in the six blood proteins mentioned above among four groups of samples, one those of remarkable band (tentatively designated D-band) could be observed between albumin and transferrin in two individuals obtained from Dogo. After treatment of acrynol (Moriwaki *et al.* 1974, Experientia **30**: 119), this protein remains in the soluble fraction with transferrin. SDS-acrylamide gel electrophoresis indicated molecular weight of this proteins approximately 50,000.

Antiserum against *Apodemus* serum proteins was prepared by immunizing rabbits with whole serum of *A.s.speciosus* collected from Fukuoka. Immunoelectrophoresis was conducted on 0.85% Agar gel using the antiserum. No visible difference was observed in the immunoelectrophoretic patterns for the whole sera of *speciosus* and *navigator* suggesting that the unknown D-band protein has a unique antigenicity.

Chromosome G- and C-bands were observed by trypsin method (Seabright, 1971) and BSG method (Sumner, 1972), respectively. So far, no geographical variation between Oki and Hondo populations has been detected.

The Genetic Control of Tryptophan Pyrrolase in *Drosophila melanogaster*: Mechanism of Suppression

Saburo NAWA and Masa-Aki YAMADA

The enzyme tryptophan pyrrolase, which catalyzes the conversion of tryptophan to formylkynurenine, is absent in vermilion (*v*) flies of *Drosophila melanogaster*. The Vermilion is known to be suppressed by a recessive mutation, *su(s)*[2] which is non-allelic to vermilion. Suppressed vermilion exhibits a partial restoration of tryptophan pyrrolase activity. Various models of genetic interaction by the suppressor gene in the control of *Drosophila* tryptophan pyrrolase have been proposed. Jacobson *et al.*

presented evidence that the vermilion produces an active but altered tryptophan pyrrolase which is inactivated by a specific isoacceptor form of tyrosine tRNA. This inhibition was removed by digesting the tRNA with RNase T_1. Similarily, the suppressor mutation, *su(s)*^{2}, removes the tRNA and thus allows the *v*-tryptophan pyrrolase to function.

The recessive mutant *a* of *Ephestia* also lacks the activity of tryptophan pyrrolase. Since we have used both *a* and *v* for the transformation experiment and obtained transformants in *a* but not in *v*, it is of interest to know whether the mutant *a* produces an altered but active enzyme which is inhibited by a RNA. In the homogenate of *a*, however, no appearance of tryptophan pyrrolase activity was observed by treatment with ribonuclease T_1 or pancreatic RNase. Then the activation of enzyme from vermilion, which has been used in the transformation experiment, was examined. No tryptophan pyrrolase activity appeared in the *v*-homogenate when treated with RNase. It has been known that there are two kinds of vermilion alleles, suppressible and unsuppressible. There may be a possibility that the *v* used is unsuppressible. Then, we obtained *v*; *bw*; *su(s)*^{2}, which is apparently brown eyed and has a detectable tryptophan pyrrolase activity. By the elimination of *su(s)*^{2} from this strain, a white eyed *v*; *bw* was produced which must be a suppressible vermilion allele. When the homogenate of this suppressible vermilion was treated with RNase, no tryptophan pyrrolase activity was observed. Furthermore, it was found that the homogenate of *Drosophila* adults, either wild type or vermilion, shows a strong RNase activity. In the condition used for the assay of tryptophan pyrrolase activity, almost all of the endogenous RNA were shown to be degraded in acid soluble forms by the endogenous RNase within 30 minutes. This means that if an altered enzyme is present in an inhibited form in the vermilion homogenate it should become active by elimination of the RNA without any exogenous RNase, since incubation of 2 or 3 hours is usual for tryptophan pyrrolase assay. Thus, the model which requires that the altered polypeptides produced by suppressible vermilion mutants are inhibited by the RNA which is absent in *su(s)*^{2}, finds no support in the present data.

Structural and Functional Properties of *Drosophila* and *Ephestia* Tryptophan Pyrrolase

Saburo NAWA and Masa-Aki YAMADA

The activity of tryptophan pyrrolase in the a^+/a heterozygote of *Ephestia* was super-additive, being greater than half the tryptophan pyrrolase activity in the a^+/a^+. Furthermore, when a/a extract was mixed with a^+/a^+ extract, the normal enzyme level was enhanced. This *a*-material responsible for the super-additivity was found to be a protein which has the same molecular weight as normal tryptophan pyrrolase. Exactly the same phenomenon as in *a* was observed in vermilion of *Drosophila*. Moreover, the *a*-protein of *Ephestia* was found to be able to enhance the activity of *Drosophila* enzyme. In the same way, the *v*-protein of *Drosophila* could enhance the *Ephestia* tryptophan pyrrolase. *Drosophila* tryptophan pyrrolase was fractionated in the same place as that for *Ephestia* tryptophan pyrrolase on Sephadex gel filtration, indicating that both enzymes have the same molecular weight. The *a*-protein as well as *v*-protein also showed the same molecular weight as normal type tryptophan pyrrolase. When a concentration of tryptophan pyrrolase, either of *Drosophila* or of *Ephestia*, in the assay medium was reduced, for example, in one-half, the activity decreased much less than half. When a volume of the reaction mixture for the assay was reduced in half without changing amounts of the enzyme, activity increased greatly.

These observations may be interpreted in terms of interaction between aggregate enzyme and its subunits, on the assumption that the subunits are inactive and the homomultimer composed of two or four identical subunits is the enzymatically active structure. An equilibrium of multimer$\rightleftarrows$subunits may vary according to the concentration of enzyme protein. The dilution of enzyme will bring the equilibrium to dissociate to subunits. The *a*-protein or *v*-protein will prevent the normal type of tryptophan pyrrolase, owing its molecular structure similar to the enzyme, from being dissociated to subunits, indicating the super-additive effect. These relationship between *Drosophila* and *Ephestia* protein with regard to the super-additivity suggests a close resembrance in structure between *Drosophila* and *Ephestia* tryptophan pyrrolase.

Genetic Control of Alcohol Dehydrogenase in the Japanese Species of *Trillium*.

Masaaki IHARA and Tohru ENDO

Genome constitution of the Japanese species of *Trillium* (Liliaceae) has been investigated with the aid of differential stainability in the Feulgen reaction of the somatic chromosomes. For this reaction, the root tip or ovular tissues are chilled for 2 to 4 days at 0° to 4°C. The karyotypes documented to date are K_1K_1 in *T. kamtschaticum* ($2n=10$), K_2K_2TT in *T. tschonoskii* ($2n=20$), and SSUU in *T. smallii* ($2n=20$) (cf. Haga, Cytologia **16**: 243, 1951). In investigating the mode of differentiation in biochemical traits, the genetic control of alcohol dehydrogenase (ADH) isozymes has been examined in the seeds of these three species and their F_1 hybrids.

Zymograms for each group are depicted diagramatically in Fig. 1.

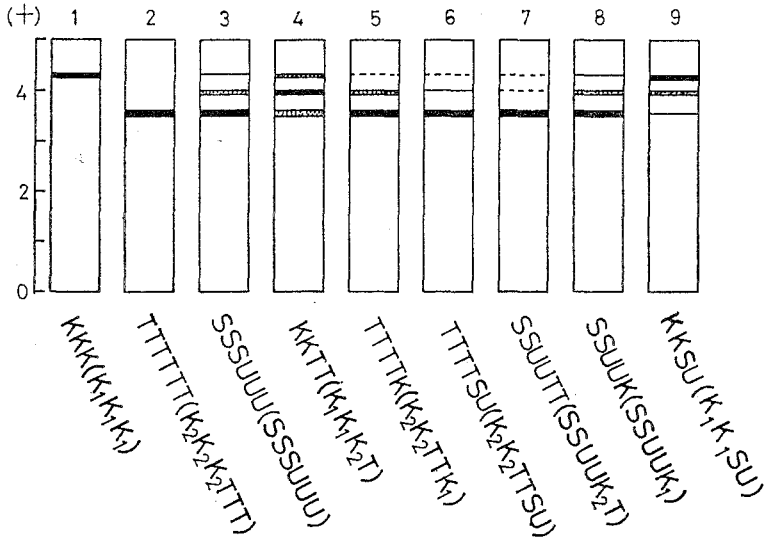

Fig. 1. ADH zymograms in Japanese species of *Trillium* (1–3) and their F_1 hybrids (4–9). The author's designation of the genomes in the endosperm and that given by Haga (1951: in parenthesis) are shown for respective zymograms. The numbers represent species and their cross: (1) *kamtschaticum* (K), (2) *tschonoskii* (T), (3) *smallii* (S), (4) K×T, (5) T×K, (6) T×S, (7) S×T, (8) S×K and (9) K×S.

The figure suggests that *T. kamtschaticum* and *T. tschonoskii* have different zymograms which are monomorphic within each species. The ADH zymogram of genome K_2 and that of genome T are identical. In *T. smallii*, we detected three discrete anodal bands, located at 3.5 cm (slow, darkly staining), 4.0 cm (middle, intermediate staining), and 4.3 cm (fast, faintly staining). Assuming that the middle band is a dimer derived from hybridization between the slow and fast moving isozymes, and that their activity ratio is $1:m$ $(1>m>0)$, we would expect a $1:2m:m^2$ ratio for the three bands. The slow and fast moving isozyme may be specified by alleles Adh^S (genome S) and Adh^U (genome U). If the m value were 0.2, the ratio would be 1: 0.4: 0.04. This is comparable to the pattern and the expected pattern even though the Adh^U allele has low activity. The action of Adh^U might be extremely low in the F_1 of *smallii* $(Adh^S/Adh^S/Adh^U/Adh^U)$ $\times$*kamtschaticum* (Adh^K/Adh^K). This may also be true for the reciprocal cross. On the other hand it gives a reaction with appreciable intensity in the F_1 of *smallii*$\times$*tschonoskii* $(Adh^T/Adh^T/Adh^T/Adh^T)$ and in this reciprocal cross. In all the F_1 hybrids, the zymographic pattern appears to be subjected to binomial expansion. An example would be in the case of TTTTK (*tschonoskii*$\times$*kamtschaticum*), $(4\tau+1\kappa)^2 \rightarrow 16\tau\tau: 8\tau\kappa: 1\kappa\kappa$, where τ and κ are ADH isozymes which are specified by Adh^T and Adh^K, respectively.

A New Method for Peroxidase Isozyme Stain

Tohru ENDO

A number of peroxidase staining methods have been reported in which the staining reaction is due either to oxidation of phenols or amines with exogenous peroxide, or by Nadi reaction of amine-phenol combinations (Endo, Bot. Mag. Tokyo, **85**: 147, 1972). The method presented here is based on a new combination of amine and phenol compounds. The reaction mixture consists of 2 mM eugenol (ca. 0.03 ml) and 0.5 mM 3-amino-9-ethylcarbazole (ca. 10 mg) which is completely dissolved in 10 ml acetone. Then, 10 ml of 1 M Tris-acetic acid buffer, pH 4.0, 1 ml of 3% hydrogen peroxide, and deionized water are added to 100 ml. Staining is best accomplished in the dark, and the peroxidase bands are stable for at least one week.

IV. DEVELOPMENTAL AND SOMATIC CELL GENETICS

Cell Lineage and Development of Chimera Hydra

Tsutomu SUGIYAMA and Toshitaka FUJISAWA

A mutant strain of *Hydra magnipapillata* (nf-1) was isolated which lacks interstitial cells and their derivatives (nerve cells and nematocytes). This strain arises by spontaneous loss of interstitial cells from its parental strain (sf-1). Reintroduction of interstitial cells from other strains into nf-1 leads to the creation of chimeric strains that consisted of epithelial cells derived from sf-1 and interstitial cells and their derivatives from other strains.

In chimeras, interstitial or epithelial cells apparently maintain very stable cell lineages; no indication was obtained that suggested interstitial cell differentiation into epithelial cells or dedifferentiation in the opposite direction during the long courses of culture of the chimeras (up to one year).

Developmental characters of chimeras were examined and compared to those of the epithelial cell (sf-1) and the interstitial cell donors. Almost all of the chimera's characters examined (graowth rate, budding rate, tentacle numbers, polyp size, regenerative capacity, etc) closely resembled those of the epithelial cell donor, but not of the interstitial cell donors. This suggests that epithelial cells, rather than interstitial or nerve cells, are the primary determinant of most, if not all, of hydra's developmental characters.

Cellular Composition of Chimera Hydra

Tsutomu SUGIYAMA and Toshitaka FUJISAWA

The hydra (*Hydra magnipapillata*) tissue consists of 6 basic type of cells; epitheliomuscular, digestive, gland, interstitial, nerve cells and nematocytes. Mutants having abnormal cellular compositions are isolated through sexual imbreeding of wild hydra. Chimeric hydra are produced by reintroducing interstitial cells from other strains into a strain (nf-1) which lacks interstitial cells, nerve cells and nematocytes.

Analyses and comparisons of the cellular compositions of all these strains revealed that the numbers of nerve or interstitial cells in the

chimeras were very similar to those in their interstitial cell donors. Since chimeras and their interstitial cell donors share the same interstitial cell lineages, this suggests that interstitial cells or their derivatives (nerve cells and nematocytes) play major roles in determining the nerve and interstitial cell levels in the hydra tissues. It is suggested that some forms of homeostatic feedback mechanisms are probably involved in regulating the levels of these cell types.

Nematocyte Differentiation in Hydra

Toshitaka FUJISAWA and Tsutomu SUGIYAMA

In hydra, the general patterns of interstitial cell differentiation into four types of nematocytes are well known. The interstitial cell divides synchronously and the resulting cells remain together in a nest as a syncytium. In each nest the cells subsequently undergo synchronous differentiation to form nematocytes of one type. However, the question remains as to what stage during cell differentiation the interstitial cells become determined to produce specific types of nematocytes. This problem of cell determination was studied statistically using mutant strains of *Hydra magnipapillata*, which have abnormal nematocyte compositions. The four types of nematocyte nests of various sizes were identified and the numbers were scored in each strain and the data were subjected to statistical analyses.

Based on the observed patterns of nematocyte differentiation, two models are put forward and compared statistically. In the first model, the interstitial cells undergo cell divisions until they reach the stage at which specific nematocyte types differentiate. In the second model, the interstitial cells are destined to differentiate into one of four types of nematocytes at an early stage, and then cell multiplication occurs. Variance test favours the first model suggesting that the interstitial cells become determined to differentiate into the specific nematocyte types after they multiply.

Pyrimidine Requirements of Cultured *rudimentary* Embryonic Cells of *Drosophila melanogaster*

Yukiaki KURODA

The *rudimentary*^{39k} (r^{39k}; 1–54.5) is a sex-linked recessive lethal gene. Homozygous or hemizygous r^{39k} embryos produced from homozygous r^{39k}

mothers died during embryonic development. When cells obtained from r^{39k} embryos prior to their effective lethal phase were cultured in K-17 medium supplemented with 0.1 mg/ml fetuin and 15% fetal calf serum, a characteristic defect was found in the maturation (chitinous pigmentation) of epithelial cells, although other types of cells from the same embryos remained normal in their growth and differentiation in culture.

It has been reported that the *r* eggs could be rescured by injecting each egg at the blastoderm stage with pyrimidine nucleosides (Okada, M., I. A. Kleinman and H. A. Schneiderman. 1974 Develop. Biol. **37**: 55). In the present experiment some pyrimidine nucleosides and intermediates in the pyrimidine biosynthetic pathway were added to the culture medium to test their repair activity of the defect of r^{39k} embryonic epithelial cells.

Uridine at 2.5×10^{-4} M and thymidine at 5×10^{-3} M were effective in repairing the r^{39k} embryonic cell defect. Carbamyl phosphate at 10^{-3} M, carbamyl aspartic acid at 2.5×10^{-4} M, dihydroorotic acid at 2.5×10^{-4} M and orotic acid at 2.5×10^{-4} M, all had repair activities on the defact of cultured r^{39k} embryonic cells. Dihydroorotic acid and orotic acid were most effective, repairing r^{39k} defect almost completely. These results suggest that r^{39k} epithelial cells may be deficient in carbamyl phosphate synthetase which is the first enzyme of the pyrimidine biosynthetic pathway.

Enhancing Effect of Cellophane Films on Chondrogenesis of Quail Limb-Bud Mesenchymal Cells in Culture

Yukiaki KURODA and Etsuya MATSUTANI

Mesenchymal cells from the limb-buds of avian embryos were known to differentiate into cartilages or muscles *in vitro*. Thus they seemed suitable material for analyzing the mechanism of differentiation of common precursor cells into the two types of cells.

In the present study a very simple culture technique with a cellophane film was found to have a marked effect in enhancing chondrogenesis of mesenchymal cells from embryonic quail limb-buds *in vitro*. Mesenchymal cells were obtained from the hind limb-buds of 3.5 day (stage 22–23) quail embryos. Drops of 0.025 ml of cell suspensions were inoculated into 35-mm petri dishes with a micropipette (Gilson, P-200). Two strips of cover glass were placed on either sides of the drop. After the petri dishes were incubated for 4 hours, 2 ml of culture medium was added to the

dishes. A cellophane film was placed on top of the glass spacers and covered with a stainless steel ring. Thus the cells were cultured in a microspace under a cellophane film.

It was found that the cellophane film markedly stimulated the process of expression of chondrogenesis of mesenchymal cells, which was estimated quantitatively by the number of cartilage nodules and the amount of toluidine blue absorbed into chondrocyte matrix. This stimulation of chondrogenesis by cellophane films was found to be due to the accumulation of some factor(s) released from mesenchymal cells under cellophane films.

Comparative Study on Mutagenic Activity of Various Agents in Cultured Human Diploid Cells

Yukiaki KURODA

In our previous works, the mechanism of mutation induction at the 8-azaguanine (8AG) resistant (HGPRT deficient) locus by ethyl methanesulfonate (EMS), furylfuramide (AF-2), phloxine and bisulfite in human diploid cells was investigated.

In the present experiment, *o*-phenyl phenol (OPP), which is a fungicidal substance for citrus fruits, and X-rays, which are physical agents for comparison, were examined for their mutagenic activities in human diploid cells.

OPP had a cytotoxic effect on human diploid cells at a concentration of 10^{-6} M for 2 hour-treatment and at a concentration of 3×10^{-7} M for 14 day-treatment. Cells were treated with OPP for 2 hours, cultured in normal medium for the mutation expression time of 48 hours, and the number of 8AG resistant cell colonies formed in the presence of 30 μg/ml 8AG was scored. No significant increase in the induced mutation frequency was observed in cells treated with OPP at various concentrations from 10^{-8} to 10^{-6} M.

When cells were exposed to 0 to 1,000 rads of X-rays, the D_0 value calculated from a dose-response curve for X-rays was 200 rads. The frequency of 8AG resistant mutations increased rapidly as an increase in the X-ray doses. X-rays at 300 rads were most effective in inducing 8AG resistant mutations without extremely marked decreases in cell survival.

When the mutagenic activities of various chemical and physical agents on induction of 8AG resistant mutations in human diploid cells were compared

at the doses giving about 50% cell survival, the induced mutation frequencies were 24.4×10^{-5} for EMS (10^{-2} M), 58.4×10^{-5} for AF-2 (10 μg/ml), 24.3×10^{-5} for phloxine (30 μg/ml), 11.7×10^{-5} for bisulfite (10^{-2} M), 0 for OPP (3×10^{-7} M) and 17.1×10^{-5} for X-rays (100 rads).

Colony-Forming Activity of Embryonic Human Diploid Cells in Primary Cultures

Yukiaki KURODA

Cells dissociated from various organs of aborted human embryos at various gestation stages were cultured in 60-mm petri dishes in Ham's medium F12 supplemented with 15% fetal calf serum to examine their colony-forming activity in primary cultures.

Among various organs from which cells were dissociated, lung cells showed the highest colony-forming activity. Heart cells were difficult to be dissociated completely into single cells and showed a lower colony-forming activity. Skin cells required a specific procedure for their pure isolation from embryos. Liver cells and kidney cells produced the extremely low colony-forming activity.

The colony-forming activity of lung cells was largely dependent on the age of embryos from which cells were obtained. The colony-forming activity was 0.022 in lung cells from a 6-week embryo, 0.126 in those from an 8-week embryo, and 0.771 in those from a 10-week embryo. Lung cells from a 13-week embryo showed the highest colony-forming activity of 1.688 among those from various ages of embryos tested. Lung cells from embryos older than 17-weeks showed a gradual decrease in their colony-forming activity under the same conditions employed. The colony-forming activity of cells from a 24-week embryo was 0.007.

Lung cells dissociated from a 10-week embryo were cultured in TD-40 flasks in primary culture, and then inoculated in petri dishes in secondary culture. The colony-forming activity of cells in secondary culture was 1.337, which were twice as high as that in primary culture (0.771). When lung cells cultured in TD-40 flasks in primary and secondary cultures were inoculated in petri dishes in tertiary culture, their colony-forming activity was 2.577, which were again twice as high as that in secondary culture. Results indicated that embryonic human lung cells showed a rapid increase in their colony-forming activity in successive subcultivation for their

initial culture period.

Fundamental Research on Genetic Monitoring of Environmental Mutagens by Using Cultured Human Cells

Yukiaki KURODA

There are some advantages in the procedures using cultured human somatic cells for monitoring genetic effects of environmental mutagens: direct effects of the mutagens on human cells could be quantitatively detected within a relatively short period under strictly controlled conditions.

Thirty human embryos were obtained by artificial abortions in seven maternity hospitals in Misima. They were at various ages of gestation from 6 to 24 weeks. Lung cells dissociated from embryos at various stages were cultured for one to three weeks in 60-mm petri dishes at 37°C in the presence and absence of 30 μg/ml 8-azaguanine (8AG). The number of colonies formed were scored and the frequency of 8AG resistant mutant cells present in the original cell populations of embryonic lung was estimated.

The frequencies of mutant cells were 0 in a 6-week embryo, 1.6×10^{-3} in an 8-week embryo, 1.8×10^{-3} in a 10-week embryo, 2.0×10^{-3} in a 13-week embryo, 7.9×10^{-3} in an 18-week embryo and 15.0×10^{-3} in a 24-week embryo. If these frequencies (y_i) are supposed to increase linealy with an increase in the age (t_i) of embryos, they are expressed as a simple formula: $y_i=at_i$. By using above data with estimated variances, the value of a was calculated to be 0.14×10^{-3}. It was found that the calculated frequencies of mutant cells in each age of embryos by using this a value coincided well with the observed frequencies in the clonal cultures. This indicates that this procedure may be useful for monitoring somatic gene mutations in human environment.

Isolation and Characterization of X-linked Maternal Effect Lethal Mutants in *Drosophila melanogaster*

Masa-Aki YAMADA and Saburo NAWA

In higher organisms, the cytoplasm of egg is produced by the female genome. The cytoplasm of egg has an essential role for the early development of the zygote. The cytoplasmic defective mutants are considered to be useful for the investigation on the interactions between the egg cytoplasm

and the zygote nucleus during the early development.

After the males of wild type fly (Oregon R) were fed on 2.5×10^{-3} M EMS in 1% sucrose solution for 24 hours, female sterile mutants on X-chromosome were isolated by Muller-5 method. From the many fs(1) mutants, 26 strains of the maternal effect mutants were picked up and divided into 16 complementation groups. All of the strains produced apparently normal eggs in shape which were unable to develop into larvae even when fertilized with wild-type sperms, and the development of the embryos stopped at various stages of embryogenesis. In some cases, the abnormality of embryogenesis also was observed at the stage of the cellular blastula or inversination.

It was examined in 7 mutants (fs(1) MY-13, 18, 131, 152, 160, 170 and 335) whether the embryos were rescued with the wild type cytoplasm injected by the microinjection method. The cytoplasm of the wild type eggs was injected into 300–600 eggs of each mutant and it was examined whether larvae could hatch. In the most of mutants, the embryos were not rescued, but in fs(1) MY-18, 13 larvae hatched from 598 eggs injected with the normal cytoplasm. In the control experiment, when eggs were injected with fs(1) MY-18 cytoplasm, no larvae were obtained. Thus, it is likely that a component of cytoplasm from normal eggs, but not from eggs of fs(1) MY-18, can rescue the developmental defect in the mutant. There is no information about the identity of this component at present. Experiments for the identification of the component are now in progress.

On the Number of Mitotic Cleavage Division Times in the Silkworm (*Bombyx Mori*)

Yoshiki OHTSUKI[1], Toshio KITAZAWA[1] and Akio MURAKAMI

Although a number of papers on the embryogenesis in the silkworm have been published, knowledge regarding the number of mitotic cleavage divisions in eggs is meagre. Ten cyclic changes in radiosensitivity with reference to embryonic lethality in accordance with the development of embryos were already observed and suggested that the number of synchronous cleavage division cycles occurred at least ten times. In addition to these synchronous divisions, a few cleavage divisions are required to complete

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blastulation. The total number of cleavage divisions required would be at least twelve to sixteen until the blastulation. After 10 or 11 cleavages, the nuclei reach the periphery of the egg and a blastula is formed. It is a well known fact that no cleavage division takes place in eggs from the completion of blastulation to gastrulation corresponding to the egg ages from 17 to 48 hrs after oviposition. Accordingly, it may be easy to calculate the number of cleavages that occur until the blastulation by counting the total number of cleavage nuclei or cells in the blastula. This report aimes histologically to calculate the total number of cleavage divisions.

Eggs of the *C108* bivoltine race were used for the present experiment. Eggs deposited within ten minutes were kept at 25°C after oviposition for observations. The number of nuclei was counted in the eggs at 13 and 72 hrs after oviposition to record the number of mitotic cleavage divisions for blastoderm formation. Histological preparations of the 13-hr-old eggs were made by a usual paraffin method and stained in Heidenhain iron hematoxylin and the nuclei in the preparation were counted. The 72-hr-old eggs (or just before the completion of pre-dormant stage) which had already completed nuclear divisions were dissected in a mixture of 0.5% acetic acid, glycerin and distillated water (dw) (9: 1: 4). Embryos were stained with acetocarmin (saturated solution of acetocarmin 1: dw 4) for 10 min on a glass printed the sectional pattern and covered with a thin glass and pressed softly to separate each cell. For both egg stages, the number of nuclei in all fields of micrographs of one preparate was counted.

The result of experiments indicated that the average number of nuclei in the 13-hr-old egg calculated was 1,985, of which 697 were destined to become the nuclei of embryonic and amnion cells and the rest of nuclei become either serosa or yolk cells. In the embryo of 72-hr-old eggs, the number of nuclei counted was 12,668.

From these findings, it can be said that eleven divisions occur until 13 hrs after oviposition at 25°C and thereafter, at least, five more divisions are required. Accordingly, the products of 16 mitotic cleavage divisions ought to form the embryo (and/or blastoderm) with more than 10,400 but less than 20,800 nuclei.

Mosaicism of Ganglion in the Silkworm (*Bombyx Mori*)

Motoya KATSUKI[1], Itaru WATANABE[1] and Akio MURAKAMI

It is worthwhile to knwo how to control genetically the differentiation of the nervous systems in higher organisms. Since the nervous system of the silkworm is composed of only 13 pairs of ganglia from brain to posterior abdomen and simple in their networks, it forms suitable material for research this field. In addition to these advantages, it has been observed independently by Murakami (unpublished observation) and by Tamazawa (unpublished observation) that the color of ganglia of wild-type stocks is black or redish-black, while that of egg(or eye)-color mutants, such as *pe*, w_1 and w_2, is milky white.

In this report, we have tried to investigate the developmental relationship between these two traits, egg (or serosa membrane cell)- and ganglion-color by using the *pe* gene. Egg-color mosaics were obtained by supercooling treatment. The wild type (*C108*; pe^+) were mated to the egg-color mutant male homozygous for *pe* gene and three hours after ovipostion, F_1 eggs (pe^+/pe) were cooled at $-10°C$ for 24 hours and then incubated at 25°C. Six hundred and five egg-color mosaics having yellow (*pe*) and black (pe^+) serosa cells out of approximately 10,000 eggs tested were detected. Among the mosaics eggs 41 hatched as larvae. For the convenience of experiments, the larvae were separated randomly into three groups: 22 larvae were reared to the 5th instar 8 days and they were killed in 80% alcohol and dissected to observe the color of ganglia under an ordinary binocular microscope. The others were furthter incubated to either pupal or adult stage. Although the ganglion in larvae was clearly visible, in both pupal and adult stages the color was very faint so it could not be clearly distinguished whether the color of ganglia is redish-black or white. The result of the observations on the ganglion of the 5th instar larvae has been summarized in Table 1. As can be seen in the table, eleven egg-color mosaics have had wild-type redish-black (pe^+) ganglia. The other 11 larvae have had mosaic colored ganglia, three of them showed mosaic ganglia as one half was redish-black (pe^+) and the another half was white (*pe*) in one side of a pair of the ganglion: two individuals had mosaicism in the right side ganglion and one in the left side one. It

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Table 1. Mosaicism of ganglia in the 5th instar larva

Individual	B	Sg	t1	t2	t3	a1	a2	a3	a4	a5	a6	a7	a8	No. of larvae
1–11	+	+	+	+	+	+	+	+	+	+	+	+	+	11
12, 13	m	+	+	+	+	+	+	+	+	+	+	+	+	2
14–16	m	m	m	m	m	m	m	m	m	m	m	m	m	3
17	+	+	m	m	m	m	m	m	m	m	m	N	N	1
18	m	m	m	m	m	+	+	+	+	+	+	+	+	1
19	+	+	m	m	m	m	+	+	+	+	+	+	+	1
20	m	m	+	m	+	m	+	+	+	+	+	+	+	1
21	m	m	m	m	m	m	m	m	m	+	+	+	+	1
22	m	m	m	m	m	m	m	m	m	m	m	—	—	1

Abbreviations: B, brain; Sg, subesophagalganglion; t, thorax; a, abdomen; +, redish-black (*pe*$^+$); —, white (*pe*); m, mosaics having redish-black and white parts in one of a pair of the ganglion.

was also observed that about 80% of ganglionmosaics appeared on only one side of a pair of the ganglia.

In summary, approximately 50% of the egg-color mosaics detected were also ganglion-color mosaics. The ganglion-color mosaics appeared randomly for each ganglion, while mosaics having for both side of a pair of ganglia were not found.

Restriction of Differentiation of Pluripotent Teratocarcinoma Cells in Some Allogeneic Strains of Mice

Takehiko NOGUCHI

Although antigenic status of the early mammalian embryos is not at present well difined, some antigens have been proved to appear at specific stages of development. Searl *et al.* (1974 Transplantation **18**: 136) reported that development of blastocysts was inhibited if they were transplanted into host mice differing from blastocyst donors either at combined H-2 and non H-2 loci, or at non H-2 loci alone. These facts imply that immunological barriers may be useful for analysis of early mammalian development. But collecting normal embryos in large numbers is not easy. In order to overcome this difficulty embryoid bodies, the ascitic form of murine teratocarcinoma, were used as the substitutes for normal early embryos because they are more easy to obtain in large numbers, and because early phase of their differentiation mimics that of the normal

embryos.

In order to see whether embryoid bodies are inhibited in their development in allogeneic hosts as in normal blastocysts, embryoid bodies of a pluripotent teratocarcinoma cell line originated from a graft of 6 day embryo of 129/Sv-SlCP strain were transplanted into the peritoneal cavity of some allogeneic hosts. These hosts were B10/Sn, B10.129(6M)/Sn and B10A/SgSn. All these mice share the same set of non H-2 histocompatibility antigen loci which are different from those of 129/Sv-S1CP.

When about 5×10^4 embryoid bodies were injected into B10/Sn males whose H-2 haplotype is H-2^b, a little different from that of 129/Sv-S1CP (H-2^{bc}), they did proliferate and formed solid tumors. About two months were required for killing hosts. It was roughly twice as long as in the syngeneic males. Embryoid bodies looked normal as far as light microscopic observation was concerned. Solid tumors were found in the similar sites as in syngeneic mice, mainly on the adipose tissues adherent to the pancreas, the mesentery and the testis. Histological examination of these solid tumors revealed that they were composed mainly of cells resembling to embryonal carcinoma cells or to embryonic ectodermal cells. At a low frequency rosette like structures of ectoderm-like cells could be observed. Many dying cells were present in between the embryonic cells. Well differentiated tissues were rarely found. Embryoid bodies were alive and capable of proliferating even after 3 successive transplantations into B10/Sn males at intervals of about 50 days. If they were back-transplanted into syngeneic 129 males at every end of the transplantations, they formed solid tumors containing various kind of tissues.

When embryoid bodies were transplanted to B10.129(6M)/Sn males which have the same H-2 haplotype as 129 males (H-2^{bc}), similar results were obtained as in B10/Sn mice. But the rosette structures of ectoderm-like cells o cured a little more frequent in this strain.

When embryoid bodies were injected into B10A/SgSn males which have more desparate H-2 haplotype(H-2^a) from 129 than B10/Sn, abdominal swelling due to increase in ascites fluid did not occured even 2 months after the injection. Embryoid bodies were found as whitish materials in between the viscera. Solid tumors remained very small. Many of them were under rejection. Embryoid bodies which attached to the adipose tissues were also being rejected.

Important points suggested by this experiment are (1) embryoid bodies

may survive and multiply for a long period of time in certain allogeneic mice, (2) differentiation of embryoid bodies may be restricted in allogeneic mice, (3) the extent of the restriction may depend on differences of the hosts from teratocarcinoma cells at H-2 as well as non H-2 histocompatibility antigen loci.

Introduction of t^{w5} and t^{w18} Tailless Mutations into LT/Sv Inbred Mice with a High Incidence of Ovarian Teratomas

Takehiko NOGUCHI

Recessive lethal tailless mutations cause death of homozygous embryos at characteristic stages. If teratocarcinoma cell lines with these mutations can be obtained, they may be useful materials for the analysis of early development of mice.

Ovarian teratomas of LT/Sv mice derive from parthenogenetically activated oocytes. Most of the ovarian teratoma cells are known to be diploid. Recent study of isozymes of teratomas obtained from a recombinant inbred strain mice produced from LT/Sv and C57BL/6J revealed that oocytes giving rise to teratomas had completed the first meiotic division (Eppig, J. *et al.* 1977, Nature **269**: 517). If LT females are heterozygous for a *t* allele, the genotype of their ovarian teratomas or teratocarcinomas are expected to be $+/+$ or t/t. Teratocarcinomas with t/t may, therefore, be obtained from $+/t$ LT females if the t/t parthenonts survive upto the stage when the mother cells of the embryonal teratocarcinomas appear. At present the mother cells are believed to be the totipotent embryonal ectoderm cells of the early egg cylinder stage embryos.

In the present experiment t^{w5} and t^{w18} were chosen. Embryos homozygous for t^{w5} and for t^{w18} are known to show abnormalities at mid egg cylinder stage and at primitive streak stage respectively. Developmental potentiality of the hypothetical t^{w5}/t^{w5} teratocarcinomas would be null. The hypothetical t^{w18}/t^{w18} teratocarcinomas would contain tissues derived from ectoderm and endoderm because t^{w18}/t^{w18} embryos are known to die due to the inability to form mesoderm.

LT/Sv females were mated with T/t^{w5} and T/t^{w18} males. The offsprings with normal tails ($+/t$) were chosen, and backcrossed to LT females which show 50% incidence of ovarian teratomas. In subsequent backcrossing the presence of *t* was checked by intercrossing the offsprings to $T/+$ mice.

Until now $+/t^{w5}$ offsprings at 5th backcross generation (B_5) and $+/t^{w18}$ at B_2 were obtained. Seventeen $+/t^{w5}$ females at B_4 and at B_5 were collected and autopsied at the age of 3 months. Three had benign teratomas. This low incidence may be due to the inability of the t^{w5}/t^{w5} parthenonts to develop. No teratocarcinomas was obtained yet.

Mice carring t^{w5}, t^{w18} and *T* were kindly gifted by Dr. K. Yanagisawa in the Mitsubishi-Kasei Institute of Life Sciences.

Abnormality in Primordial Germ Cell Proliferation in 129/ter-Sv Inbred Mice with a High Incidence of Testicular Teratomas

Takehiko NOGUCHI

In 129/terSv mice about 30% of the males develop congenital testicular teratomas (Stevens, L. C. 1973, J. Natl. Cancer Inst. **50**: 235). These tumors derive from primordial germ cells. Proliferative pattern of PGC in the fetal testes of this inbred strain was studied in comparison with those of 129 substrains with much lower incidences of the tumor.

Total number of dividing PGC estimated with hematoxiline-eosin stained serial sections of the fetal testes were ploted along the developmental stages from 12 days to 16 days of gestation. In substrains with tumor incidence of less than 1% (low strains) it made a curve with a peak at 13 days and with a foot reaching to zero level at 15 to 16 days of gestation. However the pattern of 129/terSv differentiated from that of the low strains. First at the peak region many plots of total counts of dividing PGCs scattered below the peak of the low strains. Most of the *ter* testes showed total counts level of about 2/3 of that of the peak. This reduced numbers of dividing PGCs is attributable to the reduced numbers of total PGCs in *ter* testes. About 1/5 of *ter* testes showed count levels of only 1/3 to 1/5 of that of the peak. These testes had so much reduced number of total PGCs that they looked like testes of embryos carring homozygous steel mutation which cause severe depletion of PGCs.

Second abnormal point was that the timing of entering in mitotically quiescent stage of PGC was shifted to older stage. Thus many plots scattered above the foot of the low strains. This phenomenon seems to be well corelated with teratocarcinogenesis in *ter* testes. Testes with abnormal numbers of dividing PGCs at 16 days had about 8 times as much tendency to have teratoma foci as testes with normal numbers. Further

16 day testes with teratoma foci were found to have smaller total seminiferous tubule volume than testes without teratomas. As the seminiferous tubules at this stage are filled with PGCs, smaller tubule volume indicates smaller total PGC numbers. Thus the partial depletion of PGCs may also be linked to teratocarcinogenesis.

When genital ridges from male 129 embryos are transplanted into adult testes, they develop into testes and about 80% of them have teratomas. Histological examination of these grafts indicated that marked reduction of PGC in the developing seminiferous tubules occured due to the death of PGCs. Mitotically active stage of PGC were protracted remarkably.

Partial depletion and protraction of the mitotically active stage of PGC in the developing seminiferous tubules may, therefore, have relevance to teratocarcinogenesis in the testes of 129 mice.

This work was started in the Jackson Laboratory (Maine, U.S.A.) in 1975 under the sponcership of Dr. L. C. Stevens, and was continued in this Institute.

V. CYTOGENETICS

Does the Potential of Unscheduled DNA Synthesis of Mammals Correlate with Their Life-Span?

Hatao KATO

Hart and Setlow (PNAS **71**: 2169, 1974) reported that abilities of diploid fibroblasts derived from seven species of mammalian species to perform unscheduled DNA synthesis (a measure of excision-repair) after UV-irradiation were correlated well with the life-span of the speices. Based on an assumption that UV is a good model insult which mimmics the normally occurring DNA damage, they have postulated that the potential for repair of DNA damage would be one of crucial factors determining life-span of mammals. However, several basic questions seem to remain unanswered. Firstly, the number of species studied may be too small to generalize their results to *Mammalia* as a whole, which contains nearly 5,000 species. Secondly, although they regarded that almost all mammalian diploid cells have similar DNA contents, recent studies indicate clearly that this is not correct. The ability of unscheduled DNA synthesis is assessed by the number of silver grains per non-S nucleus in autoradiographs but not per unit amount of DNA, hence neglection of the genome-size difference among species may cause a serious bias concerning the true repair potential. These questions prompted me to re-investigate the ability of unscheduled DNA synthesis following UV-irradiation of 34 mammalian species in reference to their life-span and genome size.

Results are shown in Fig. 1. Cytophotometrical measurement of the DNA content of cultured cells revealed that the genome sizes of mammals here studied varied from 58% to 141% of that of human. Refering to these results, observed values for unscheduled DNA synthesis were corrected.

As shown in this figure, the mojority of species did not show any correlation between the repair potential and their life-span. A typical example that contradicted to the reported correlation was the cases of Primates. The maximum life-spans of the tree shrew, the crab-eating monkey and man are 5 yr, 15.5 yr and 110 yr, respectively, but all of them showed the same degree of repair potentials. Another good example was found in chiropteran species. The reported maximum life-spans of bats

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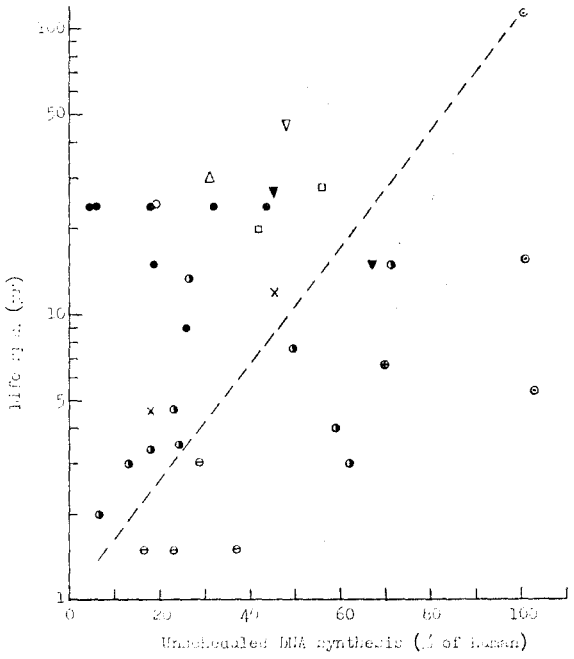

Fig. 1. Relation of repair potential of life-span in 34 species of mammalian species. The symbols denote groups of species as follows: ×, Marsupialia; ⊖, Insectivora; ●, Chiroptera; ⊙, Primates; ○, Edentata; ⊕, Lagomorpha; ◑, Rodentia; △, Cetacea; □, Carnivora; ▽, Perissodactyla; ▼, Artiodactyla. A broken line indicates a presumptive correlation between life-span and the ability of unscheduled DNA synthesis.

range from 15 yr to 24 yr. The abilities of their fibroblasts to perform unscheduled DNA synthesis varied from 5 to 40% of that of human and this was almost the same as that of insectivores that are known to be the most short-lived mammals (1.5 yr). The repair potential of the giant anteater (Edentata) was also found to be about 20% of that of human, whereas its life-span is reported to be 24.5 yr. It is of interest to note that all of these animals are phylogenetically related. Among rodents, repair potentials varied greatly independent upon their life-spans but tended to be similar within a Family.

In the present study, five (shrew, mouse, rat, golden hamster and man) our of seven animals (the former plus cow and elephant) utilized by Hart

and Setlow were examined. Interestingly enough, uncorrected values for their repair potentials could be plotted on or near the presumptive correlation line. The values for two artiodactyls other than the cow were also distributed near this line. These findings seem to suggest that the difference in experimental systems between the present and the previous reports as described in the legend to figure does not largely affect the results of assessment of repair potentials, and may imply that the excellent correlation between the extent of unscheduled DNA synthesis and life-span of the species observed by the previous workers could be a spurious one.

Repair potentials were determined by using diploid fibroblasts mostly derived from lung tissues of adult animals whose exact ages were unknown. Cultures were used for experiments at their earlier passages. For some of animals, cultures were established from both lung and epidermis. The extents of unscheduled DNA synthesis in the skin and the lung cultures were found to be comparable. To detect unscheduled DNA synthesis, exponentially growing cells on coverslips were treated with 0.1 mM FUdR for 1 h, washed with buffered saline and exposed to UV light (254 nm) at a fluence of 10 Jm^{-2}. Cells were then incubated for 4 h in medium containing 5 μCi/ml ^{3}H-thymidine (spec. act. 12.6 Ci/mM) and processed for autoradiographic preparation immediately thereafter.

Temperature Depandent Formation of Sister Chromatid Exchange

Hatao KATO

There is evidence that sister chromatid exchange (SCE) in mitotic chromosomes of higher organisms occurs spontaneously. In general, its incidence in cultured mammalian cells ranges from 2 to 6 per metaphase per cell generation at 37°C, and this frequency is at least 100 times higher than that of spontaneous chromosomal aberrations. Its constant occurrence indicates that SCE is not a sporadic event but a phenomenon occurring as a function of time under physiological conditions. Furthermore, it is assumed that any causes trigerring a process of SCE formation would be intrinsic. Had the cause be intrinsic, either the occurrence of the cause *per se* or its biological consequence might be influenced largely by temperature. Based on this consideration, effects of temperature on the incidence of SCE was studied by using cultured Chinese hamster cells.

Cells were subcultured into 25 cm^2 plastic culture flasks at a concentra-

tion of 10^5 cells per flask. One day after subculturing, flasks were transferred to light-proof incubators adjusted at various temperatures. After an adaptation period of 24 h to each temperature 0.25 μg/ml bromodeoxyuridine was added to cultures, which were then grown for two rounds of cell cycle before fixation for chromosome preparation.

The incidence of SCE in these cultures varied greatly dependent on the incubation temperature. The SCE frequency at 31°C was 2.6 per cell, while it increased gradually with increasing temperature to reach 13.9 per cell at 42°C. The frequency of cells having chromosomal aberrations such as chromatid gaps and breaks was found to be 2% at temperature between 31°C and 39°C but it increased abruptly at 40°C and above. At 42°C all metaphases were found to possess aberrant chromosomes.

The temperature dependence of the SCE formation is shown in the form of an Arrhenius plot in Fig. 1. The SCE frequency at each temperature could be plotted nicely on a regression line which showed a break at 39°C. The presence of this break seems to imply that the SCE formation cannot be achieved by a single chemical process and that at least one of processes might function to enhance SCE formation at temperature higher than 39°C.

Analyses of cell kinetics of cultures grown at various temperatures indicated that the length of the DNA synthesic period (S) was shortened gradually as the incubation temperature was raised from 31°C to 39°C. However, above 39°C, it was elongated remarkably. It is very likely that the elongation of S is due to a disturbance in the process of DNA replication, and that the disturbance is responsible for the promotion of SCE formation.

In order to detect a cellular phase at which the temperature treatment was most effective in enhancing SCE formation, cultures were synchronized by the mitosis selection method at 37°C and exposed to 41°C for various time lengths during the first cell cycle. Results indicated that a significant increase in the SCE frequency was detectable only when the high temperature treatment was given during S phase. This finding also indicates that DNA replication is intimately related to the mechanism for spontaneous SCE.

The temperature dependent increase of the SCE frequency clearly shows that the effect of temperature was not only to speed up an SCE formation process but also to increase the number or amount of the intrinsic causes that trigger the SCE process. Furthermore, the break in the regression line

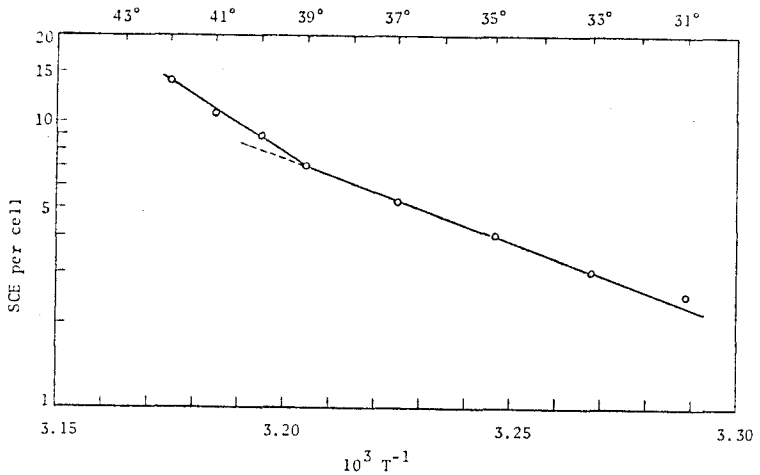

Fig. 1. Arrhenius plot of frequencies of SCE in Chinese hamster cells.

in the Arrhenius plot at 39°C suggests strongly that a disturbance in the DNA replication process at temperature higher than 39°C might exert an additive effect on SCE formation.

A Simple Technique for Observation of Sister Chromatid Exchange *In Vivo*

Naotoshi KANDA and Hatao KATO

In vivo observation of sister chromatid exchange by the BUdR-labeling method is hampered greatly by a rapid degradation of bromodeoxyuridine (BUdR) within the body of the host animal. To solve this problem, BUdR is usually administered to the host either by repeated intraperitoneal injections at an hourly interval for a period covering a whole cell cycle or by continuous infusion through the tail vein for one or two rounds of cell cycle using a special pumping device.

In an attempt to avoid these laborious procedures, we have developed a simplified technique that consisted of a single intraperitoneal injection of BUdR absorbed on activated charcoal and assured continuous supply of BUdR to target cells throughout two rounds of replication cycle. In brief, BUdR aqueous solutions were first prepared at various concentra-

tions, and to these solutions, neutralized and sterilized activated charcoal was added at a concentration of 100 mg/ml. 1 ml or 0.5 ml of the BUdR-activated charcoal suspension was injected to each animal.

This technique was applied to observation of sister chromatid exchange in tumor cells proleferating in the peritoneal cavity of the mouse and to mouse spermatogonial cells. In both types of cells, the minimum dose of BUdR required for sister chromatid differentiation was found to be 5 mg/body (6–8 week-old C3H mouse), and average exchange frequencies were 4.5/cell in tumor cells and 1.8/cell in spermatogonia. The latter value was identical to those obtained by using other techniques for the mouse spermatogonia. Because of its simplicity, this technique seems to be useful to study effects of chemical mutagens on germ-line cells as well as to study meiotic processes in various animals.

Some Genetical Aspects on Supernumerary Chromosomes in the Black Rats

Tosihide H. YOSIDA

Presence of supernumerary of B-chromosomes has been described by Gropp *et al.* (1971), Young and Dhaliwal (1972), Raman and Sharma (1974) and Yosida (1977) in the Asian type black rats with $2n=42$. According to these authors the number of supernumeraries varied from one to four, and their shapes were usually metacentrics which were morphologically indistinguishable from the regular metacentric autosomes. Supernumeraries with the same morphology as found in the Asian type rat were also observed in the Ceylonese ($2n=40$) and the Oceanian type black rats ($2n=38$) (Yosida 1977). Genetic surveys of supernumeraries in mammalian species are rare, and no report on the genetics of supernumeraries in the black rat.

The black rats used in the present study were originally obtained from Thailand and were bred for two years in this laboratory. Supernumeraries were often observed in the offspring of these rats. To know the mode of inheritance of the supernumerary chromosomes, parents with one supernumerary were mated and 37 offspring from 7 litters were obtained. Among them 31 rats had from 1 to 3 supernumeraries; 11 rats had 1 supernumerary, 14 had 2 and remaining 6 had 3 supernumeraries. From the mating of a female with 2 supernumeraries and a male lacking a super-

numerary, 17 offspring from 5 litters were obtained. Among these rats, 13 had from 1 to 4 supernumeraries. In the third case 12 offspring (3 litters) were obtained from a mating between a female with three supernumeraries and a male without the supernumerary. Among them 10 rats had from 1 to 6 supernumeraries.

According to Raman and Sharma (1975) the supernumeraries in the black rat do not show conjugation in the meiotic-I. From the above data it is suggested that the supernumerary would be moved to either one pole or the other without division in meiotic-I and that the non-disjunction of the supernumeraries would occur at random in the meiotic-II phase. Thus the number of supernumeraries included in the offspring is higher as shown by the above matings. Generally speaking, the number of rats with few supernumeraries was considerably larger than the expected one, while the number of the rats without the supernumeraries was smaller. From these results it can be said that black rats with only a few supernumeraries have a selective advantage over those without supernumeraries.

Some Genetical Aspects of C-band Polymorphism in the Black Rats

Tosihide H. YOSIDA and Yuriko OCHIAI

A remarkable polymorphic variation of the heterochromatic C-bands in the Japanese black rat (*Rattus rattus tanezumi*) has been reported by Yosida and Sagai (Chromosoma **50**, 283–300, 1975). The present study deals with some genetic investigations in the C-band polymorphism of these animals bred in the laboratory. In 62 black rats examined, chromosome pairs no. 1, 3, 7, 8, 10, 11 and 13 were polymorphic with regard to the presence or absence of the band. In pair no. 1, 45.2 per cent showed C-bands in both members of the pairs, 40.3 per cent were heteromorphic due to the presence or absence of the band, and in the remaining 14.5 per cent C-bands were absent in both members of the pair. Pair no. 1 in the Japanese black rat was polymorphic for acrocentrics and subtelocentrics as reported previously. The C-band in pair no. 1 was always observed in acrocentric chromosomes.

A mode of inheritance of the C-band patterns from the parents to the offsprings was examined in 24 rats (5 litters) derived from 5 different set of parents. In a total of 288 chromosomes observed in the 24 offspring the C-band positive and negative types were segregated 148: 140. This value

closely corresponds to the expected one of 150.0: 137.5, and indicates that the C-band polymorphism of the black rat is exactly transferred to the offspring by the Mendelian segregation ratio.

For the development of the large metacentric chromosome in the Chichijima black rats, the following two explanations are possible: 1) Robertsonian fusion between pairs no. 11 and 12 independently occurred from the Ceylonese and the Oceanian type rats with large metacentrics by fusion of these chromosomes. 2) Ceylonese or Oceanian type black rats migrated to this island resulting in hybridization with the Asian type rat. A conclusive statement on the origin of the metacentrics however, will require further studies using a larger number of specimens.

Robertsonian Fusion of Acrocentric Pairs no. 11 and 12 in Black Rats Obtained from Chichijima Island, Japan

Tosihide H. YOSIDA

Three specimens of the black rat, *Rattus rattus*, were obtained from Chichijima, one of the Ogasawara islands of Japan by courtesy of Mr. Mitsuru Minezawa of the Institute of Primates, Kyoto University. Among them, one male (a) had 42 chromosomes, the typical Asian type idiogram. Two others, one female (b) and one male (c) were found to have 41 chromosomes. Their karyotypes contained one large metacentric chromosome. All 10 cells from the b and c showed the same metacentrics. Karyotype analysis suggested that Robertsonian fusion between members of pairs no. 11 and 12 resulted in the formation of the metacentrics. Unpaired acrocentrics 11 and 12 seemed to be homologous to the long and short arms of the metacentrics. G-band analysis clearly showed that the large metacentric chromosome consisted of the Robertsonian fusion of chromosomes 11 and 12.

As previously reported by the present author, chromosome pairs no. 1, 9 and 13 in the Asian type black rats are polymorphic with regard to the acrocentrics and subtelocentrics. The three Chichijima black rats also showed a polymorphism of a similar nature for these chromosomes. Yosida and Sagai (1975, Chromosoma, **50**: 283–300) demonstrated that the Japanese balck rat (*R. rattus tanezumi*) was distinguishable by having C-band polymorphism for the presence or absence of these bands. The present materials were also polymorphic by showing C-bands of a type similar to

other *tanezumi* rats.

Frequent Occurrence of Sex Chromosome Anomalies in F_2 Hybrids between Geographical Variants of the Black Rats

Tosihide H. YOSIDA

By their karyological characteristics, black rats (*Rattus rattus*) may be classified into three geographical types, Asian ($2n=42$), Ceylonese ($2n=40$), and Oceanian ($2n=38$). In any combination of these three types, F_1 hybrids are easily produced, but they seem to be semisterile, except for those between Ceylonese and Oceanian types. Among the F_2 hybrids between the Ceylonese and Oceanian types, rats with sex chromosome anomalies have often observed. When the present author (Yosida 1977, Cytogenet. Cell Genet. **19**: 262–272) first observed the XO female in the F_2 hybrids, the occurrence of this abnormality was assumed to be sporadic. In further observations another XO female and an XXY male were found in the same hybrids. The sex chromosomal abnormalities as seen in the F_2 hybrids were never observed in non-hybrid offspring in Ceylonese and Oceanian type black rats, so far as the observations were taken on our breeding colonies.

All F_1 hybrids obtained by mating Oceanian and Ceylonese type black rats invariably possessed 39 chromosomes. By mating the F_1 hybrids 22 F_2 offspring (11 females and 11 males) were produced. Among them 6 (2 females and 4 males) were Oceanian type, 13 (8 females and 5 males) were the F_1 type, and the remaining 3 (1 female and 2 males) were of the Ceylonese type. The segregation ratio of these three types was very similar to the theoretical one (5.5: 11.0: 5.5). Among the F_2 offspring both females with the Oceanian type showed the same XO in the sex chromosome complement. The one male with Ceylonese karyotype was remarkable by having the XXY. The other females and males had the normal sex chromosome pattern, XX and XY. Non-disjunction of sex chromosomes in gametegenesis of the F_1 hybrids may be the cause of the high occurrence of the sex chromosome anomalies in F_2 offspring.

Artificial Insemination between *Rattus norvegicus*, *R. annandalei* and *R. losea*

Tosihide H. YOSIDA and Choji TAYA

The results of artificial insemination between the female Norway rat (*Rattus norvegicus*) and the male black rat (*Rattus rattus*) have been previously reported. The results were that among 20 rats, in which the insemination was successfully achieved, 9 rats (45%) were successfully pregnant (Yosida and Taya 1977, Jap. J. Genet. **52**: 289–299). The average number of uterine implanted eggs per rat was 2 ± 1.2. However, all embryos always degenerated within two weeks after insemination. The present study was undertaken to determine the success rate of artificial insemination between the Norway rat and two other related species, *R. annandalei* and *R. losea*. Some spercimens of *Rattus annandalei* were originally collected in Malaysia in 1972, and a colony has been maintained in this institute for about six years. The chromosome number of this species is 42 and the karyotype is similar to the black rat. Spermatozoa of *R. annandalei* were inseminated into the female Norway rats by the same technique as that used for the balck rat. Among 28 rats which were successfully inseminated, only 5 rats (18%) became pregnant. An average of 1.7 ± 1.2 eggs were implanted in the uterus of the pregnant rats. The embryos usually perished within two weeks after insemination. Based on the above investigations it is clearly shown that the pregrant ratio by artificial insemination between the Norway rat and *annandalei* was lower that between the Norway rat and the black rat. This means that the evolutionary distance between the Norway rat and *annandalei* is greater than that between the Norway rat and black rat.

Some of male and female *Rattus losea* were obtained from Thailand in 1976, and they were bred in the laboratory for two years. This species also had $2n=42$ and a similar karyotype to the Asian black rat. Twenty four Norway rats were successfully inseminated with the spermatozoa of *R. losea*. In this fertilization, no pregnant females were obtained. In order to determine whether or not the egg of the Norway rat was successfully fertilized by the spermatozoa of *R. losea*, 6 rats were dissected on the 5th day after insemination, and their eggs were examined. In all rats, 3 to 6 eggs were obtained and among them fertilized eggs were found in 5 rats. However, they were only cell masses, whereas in the control a normal

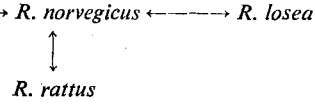

Fig. 1. An illustration of the evolutionary positions between the Norway rat (*Rattus norvegicus*) and the other *Rattus* species (*R. rattus*, *R. annandalei* and *R. losea*) as suggested by embryonic development after artificial insemination.

blastocyst was present by this time. These eggs, therefore, would not be implanted in the uterus of the Norway rat. This experiment shows that the evolutionary relationship between *norvegicus* and *losea* is more distant than that between *norvegicus* and *annandalei*. Based on the results of the artificial insemination between the Norway rat (*R. norvegicus*) and the other 3 species (*R. rattus*, *R. annandalei* and *R. losea*), the evolutionary positions seem to be as illustrated in Figure 1.

Candidates for a New Experimental Animal, II. The Indian Spiny Mouse, *Mus platythrix*.

Tosihide H. YOSIDA

Indian spiny mice, *Mus platythrix*, were originally collected in Mysor, India in 1972, and have been successfully bred in this laboratory by brother and sister matings for about 8 to 10 generations by our standard mouse breeding system. The size of this animal is slightly larger than the house mouse. The mean body weight of 19 females and 13 males, fully grown is 36.1 g and 43.0 g, respectively. Laboratory breeding of this animal is simple and the litter size is 5.4. The gestation period averages 21 ± 1 days, and the eyes open 13 to 15 days after birth.

The number of chromosomes in this species has been previously reported (Tsuchiya and Yosida, this Annual Report, **22**: 51–52, 1971). The diploid chromosome number is 26 and all autosome pairs and X and Y chromosomes are acrocentrics. Based on comparisons of chromosome G-bands and chromosome lengths between the spiny mouse and the house mouse (*Mus musculus*), it was suggested that 8 autosome pairs (no. 1 to 8) in the former species had probably occurred from the tandem fusion of several combinations of two acrocentric pairs in the latter species. Pairs 9 to 12 and X and Y in the spiny mouse seemed to correspond to some autsomal pairs and to the X and Y of the house mouse.

By having fewer and larger chromosomes, the Indian spiny mouse may prove to be more useful than the house mouse for some types of biomedical research such as cytogenetic surveys.

Difference of Nucleolar Organizer Regions in the Black and Norway Rats

Tosihide H. YOSIDA

Two species of genus *Rattus*, the black rat, *R. rattus*, and the Norway rat, *R. norvegicus*, are characterized by the same chromosome number ($2n=42$) and a similar karyotype. Based on comparative karyotype analysis of these two species, the present author (Yosida 1973, Chromosoma, **40**: 285–297) postulated that the Norway rat may have differentiated from one type of polymorphic karyotype which occurs in the Asian type black rat. In the black rat there are three geographical variants having $2n=42$, 40 and 38 (Yosida *et al.* 1974, Chromosoma, **45**: 99–109). Although the karyotypes are markedly different from each other, hybridization easily occurs between these three types. Hybrids between the black and the Norway rat, however, have never been obtained, even though artificial insemination was employed (Yosida and Taya 1977, Jap. J. Genet. **52**: 289–299).

Nucleolar organizers are known to be important structure of chromosomes in relation to the formation of the nucleolus as a site of ribosome RNA synthesis. From a comparative study of the nucleolar organizer regions of chromosomes of the black rat and the Norway rat, some evidence indicating an evolutionary relation between these species may be suggested. Observations show that both species have the silver stained nucleolar organizer regions in three chromosome pairs. In the black rat the regions are found in pairs no. 3, 8 and 13, although there are some polymorphic changes in their occurrence. The nucleolar organizer regions are found in these three chromosome pairs in all three geographic types of black rats.

In the Norway rats the nucleolar organizer regions were observed in chromosome pairs 3 and 13. This is similar to the pattern present in the black rat. However, the organizer region is not present in pair no. 8 in the Norway rat, but is observed in pair no. 12 (chromosome pairs no. 12 and 13 in the Norway rat described here correspond to pairs no. 11 and 12 in the Standard Karyotype System). This investigation indicates that the change of the nucleolar organizer regions may by important in species

differentiation.

Cytogenetical Identification of the X-Chromosome in *Bombyx Mori*

Akio MURAKAMI, Akio OHNUMA and H. T. IMAI

The karyotype in the silkworm *Bombyx mori* is $n=28$ with male homogamety (XX or ZZ) and female heterogamety (XY or ZW). The chromosomes are holocentric and small in size and do not show marked individual variations. With recent developments in chromosome observation techniques which give better resolution, it is possible to observe greate cytological details of the chromosome during mitotic as well as meiotic division stages in this insect (Murakami and Imai, 1974). However, we could not distinguish morphological differences between sex-chromosomes and autosomes. But, we have been able to recognize either bivalent or trivalent chromosome due to a differential thickness of the chromosome pairs and/or a differential chromosome pairing figure. Namely, two members (bivalent) of the trivalents are connected tightly along the entire length, while one remained single (univalent) and is often loosely coiled.

Based on these observations, we have proposed a hypothesis that when a hypo-triploid male such as a 3A+XX is obtained, it would be possible to identify morphologically the X-chromosome (as well as Y-chromosome) from autosomes. The following mating scheme has been devised to obtain the hypotriploid male. Females having a Y-chromosome translocated with pe^+ gene which is located on the 5th linkage group, $T(Y;5)$, and the 5th chromosome homozygous for *pe* (yellowish-white egg, 0.0) are crossed with tester males homozygous for *sch* (sex-linked chocolate, X-21.5) and *pe*. The resultant F_1 hybrid females are treated with CO_2 gas at egg stages from 60 to 120 min after oviposition to induce tetraploid females, $\begin{pmatrix} Y\text{——}pe^+\ pe \\ Y\text{——}pe^+\ pe \\ X\text{—}\frac{sch^+}{+}\ pe \\ X\text{—}\frac{sch}{+}\ pe \end{pmatrix}$. In this mating, it is expected that several other genotype of F_1 hybrids such as diploid, triploid silkworms and so on would appear. The female moths developed from the tetraploid eggs are backcrossed to the tester male moths. In this cross, various classes of BF_1 offspring can be detected: females hatched from wild-type (black) eggs having a chromosome constitution either 3A+XXYY or 3A+XXY and hypo-triploid males derived

from yellowish-white eggs having a chromosome constitution 3A+XX. Males in the latter case would arise due to non-disjunction of sex-chromosomes. This 3A+XX individual is the just expected hypo-triploid male.

Testes of this 3A+XX line male larvae of either 3rd or 5th instar were used for chromosome observations. The improved air-drying technique developed by Imai *et al.* (1977) was adopted for chromosome observations. Chromosome number of spermatogonial cells were analyzed for eight individuals. The number of chromosomes in 71 metaphase stages collected from 8 hypo-triploidal individuals was found to be 83 ($3n$-1). Theoretically assuming that all the autosomes in this hypo-triploid male should make trivalents, while the X-chromosomes form bivalents and that chromosome condensation between trivalents and bivalents should not remarkably differ. As a matter of course, the paired X-chromosome should be slender (2/3) in which as compared with the other trivalent autosomes (at pachytene stage). In fact, one unusual chromosome pair was observed which was slender while there were 27 thick chromosomes corresponding to trivalents. This slender chromosome pair would be the bivalent of the X-chromosomes. Chromosome size or relative percentage to the total length of the haploid set was measured using 16 well spread pachytene stages. The result obtained so far indicate that the X-chromosome is one of the member of the large chromosome group or the 3rd longer in a statistical sense.

This idea presented for the identification of the X-chromosome may be also applied to other linkage groups in *Bombyx*. At present, chromosome identification of the 5th linkage group of *Bombyx* is in progress.

VI. MUTATION AND MUTAGENESIS IN ANIMALS

An Exceptional Pattern of Mutation Spectrum Observed in the Offspring of Mitomycin C Treated Females of a Strain rb of the Silkworm

Yataro TAZIMA

The preponderance of mosaics among induced mutants has been known to be a chracteristic feature of chemical mutagenesis. When mutations were induced by ionizing radiation it was found that the number of induced mosaics increased remarkably with the progress of spermiogenesis after meiosis. Mutations induced by chemicals, however, manifest themselves as mosaics even when treatment was performed before meiosis. For instance, the proportion of mosaics is as high as 85–95% when spermatids had been treated with Mitomycin C.

The mutational response of female germ cells is quite different from what is known for male germ cells. Although the incidence of mosaics, after chemical treatment, exceeds whole-types, its proportion to total mutants is usually far smaller than that for male germ cells, i.e. 60–70%.

An exceptional case of mutation response, i.e. less mosaics than whole-types, was discovered for *rb*, a strain most sensitive to ionizing radiation. When female pupae of this strain had been injected with Mitomycin C the incidence of mosaics was less than that of whole types. Similar treatment of other strains, Kansen, Aojuku, Sekko and C108 did not show such distortion in the proportion of mosaics. Further, injection of EMS into *rb* showed no distorted mutation spectrum.

In these experiments injection was performed from 1 to 6 days before meiotic metaphase, when each chromosome were ready for meiotic division after completing the formation of two chromatids. The high incidence of whole-type mutants could, therefore, be accounted for by either one of the following alternative hypothesis (1) two helices of DNA were simultaneously affected by Mitomycin C or (2) one helix was affected and DNA replication occurred once more before the completion of meiotic division. The finding that the distortion was found in a radiation sensitive strain seems to support the former hypothesis.

Metabolic Activities of Silkworm Microsome Fraction on Some Indirect Carcinogens with Special Regard to the Differential Mutation Response Between Silkworm and *Drosophila*

Akio MURAKAMI, Michiko GOTO and Yataro TAZIMA

Indirect carcinogens thus far most intensively investigated in the silkworm are N-dimethylnitrosamine (DMN) and N-acetylaminofluorene (AAF). To those compounds silkworm (*Bombyx*) and *Drosophila* were found to respond differently as regard to mutation induction, i.e., 1) DMN is clearly effective in producing recessive lethal mutations in *Drosophila*, whereas it is not mutagenically active in *Bombyx*, 2) AAF has been reported to be incapable of producing recessive lethal mutations in *Drosophila*, although weak mutagenicity was detected by heterochromatic loci method using *bobbed*. The silkworm system clearly detects a positive mutagenicity of this compound although only at a very high dose, 500 μg/insect.

In order to reveal the mechanism(s) underlying the discordance, we investigated the metabolic activation potentiality of *Bombyx* microsome fraction using the mutagenicity to *Salmonella* as a criterion.

Microsome fraction (9000 G supernatant) was prepared from the homogenates of *Bombyx* at various metamorphosic phases and the activity was determined by mutagenicity of indirect carcinogens+microsome fraction to *Salmonella* T98 and T100 strains. The microsome fraction from the fifth instar larvae was highly active on AAF, but those from pupae and eggs were almost inactive. In contrast, the same fraction, even from fifth instar larvae, did not activate DMN.

These results seemed to suggest that the lack of mutation response to DMN in *Bombyx* could be attributed to the lack of metabolic activity on DMN in silkworm microsome fraction. However, this assumption could not apply to *Drosophila*, because *Drosophila* microsome was found to activate AAF very effectively (Brewen and Nix, 1978).

Accordingly, the above mentioned discrepancy in mutation response between *Bombyx* and *Drosophila* appears to be due to mechanism(s) other than the metabolic activation capacity detected by *Salmonella* mutagenicity testing systems.

Mutagenic Effectiveness of an Internal γ-Emitter, Tritium (4) RBE of Tritium at Low Dose-Rate Range

Yataro TAZIMA, Kimiharu ONIMARU and Yosoji FUKASE

Experiments have been continued to estimate the mutagenic effectiveness of tritiated water (THO) at low dose-rate range paying special regards to RBE. Materials and methods used for tritium experiment was almost similar to those reported previously (this Report No. 27: 62). Tritiated water was injected to wild type female pupae of strain C108 at doses of 60 and 30 μCi per pupa. Emerged females were mated to males of another wild type strain Aojuku and deposited eggs were subjected to acid treatment for artificial hatching. Average radioactivity transmitted to the deposited egg we.e 27073.8 dpm and 14100.0 dpm, from which radiation dose-rate absorved by germ cells were calculated to be ca. 7.28 and 3.77 rad/day. The duration from injection to hatching, based on which total irradiation dose was calculated, was 17.75 days. The calculated absorbed doses were 129.3 and 67.3 rad respectively.

Gamma-ray irradiation was performed using special chronic irradiation apparatus of 1 Ci ^{60}Co source. Female pupae of strain C108 were placed on rotating trays, 165 cm and 228 cm apart from the ^{60}Co source, at exactly same time when THO injection was performed and they were continuously irradiated there until emergence. Irradiation was continued even when they were mating with males of strain Aojuku, depositing and throughout egg stage until hatching except for a few hours' intermission for artificial hatching. The net exposure time for γ-rays was 16.6 days and radiation dose-rates and total doses delivered (shown in parentheses) were 11.6 rad/day (193.0 rad) and 5.8 rad/day (97.3 rad).

Those treated individuals were raised and mated to partners with double recessive markers, *pe re*, in order to assess the mutation frequency.

The results showed clearly linear relationship with increasing dose for THO group but rather obscure for γ-ray group. They were approximately represented as follows:

for irradiation of female

$$\text{THO:}\quad Y=(1.58X+62)\times 10^{-4}$$

$$\gamma:\quad Y=(0.88X+62)\times 10^{-4}$$

for irradiation of male

$$\text{THO:}\quad Y=(2.04X+171)\times 10^{-4}$$

γ: $Y=(1.14X+171)\times 10^{-4}$,

where Y denotes mutation frequency per 10^5 when absorbed energy X was given in rad.

From these results RBE's of THO were calculated to be 1.8 for both sexes.

Experiments on the Biological Effects of *Ortho*-Phenylphenol Sodium Salt for Silkworm Germ-Cells

Akio MURAKAMI, Yosoji FUKASE, Hiyorio NISHIJIMA and Michiko GOTO

Ortho-phenylphenol (OPP) has been permitted to use only as a fungicidal agent for fruit packages and/or wrapping papers, but this agent was detected in imported oranges in April, 1975. Consumer-organisations expressed their concern about it use. One report gave positive DNA damaging effects in micro-organisms (Nishioka and Ogasawara, 1977), whereas the other reports showed no evidence for any genetical or cytogenetical effects in microorganisms, mice and mammalian cultured cells (Ochi and Tonomura, 1977; Shirasu *et al.*, 1976). In view of the gravity of the problem, as well as even the remote doubt about the induction of genetic hazards by this agent is likely to endanger the human population, a detailed investigation was taken up to study the genetic and biological effects of this compound in the silkworm (*Bombyx mori* L.).

An F_1 hybrid from a cross between wild-type silkworm strains *C108* and *Aojuku* and a marker stock having double recessive egg-color genes, *pe* and *re*, were used in all experiments. *Ortho*-phenylphenol sodium salt (OPP.Na) used in this experiment was supplied by the National Institute of Hygienic Sciences as a test sample (JMS 74–20) for the Carcinogen Screening Test Programme.

Toxicity or killing test for a single dose of OPP.Na was measured as the reduction in survival (or adult emergence) ratio. LD_{50} values at various developmental stages of the silkworm were estimated from the dose-response relationships. Genetic toxicity test was measured by the dominant lethal mutation and recessive visible mutation. Statistically significant reduction in hatchability represents the occurrence of the dominant lethal mutation. The latter type mutation was measured by the egg-color specific locus test. Significant reduction in the average number of eggs deposited per female moth which had been treated or untreated with the agent and mated to untreated or treated male, respectively, indicates occurrence of

infecundity.

LD_{50} value on gram body weight basis was in the range of *ca.* 100 to 600 μg: the LD_{50} is 230 μg in a day old larvae of the 5th instar, 100 μg in 4 days old larvae of the 5th instar, 300 μg in mid-stage pupae and 600 μg in late-stage pupae. The results obtained from the dominant lethal test in spermatid and sperm following treatement of 5th instar day 3 male larvar and mid-stage male pupae with OPP.Na indicated no significant increase of this class mutation above the spontaneous level at 2.2 to 414.3 μg/g body weight. Evidence for the induction of dominant lethal mutation in pupal oocytes at the concentration tested ranging from 1.8 to 182.5 μg/g pupal weight was not observed. This fungicide is shown to be non-mutagenic with the specific locus test in both pupal sperm and oocytes between 2.2 and 182.5 μg per gram pupal weight. It should be noted, however, that OPP.Na can cause high incidence of infecundity (or fecundity was reduced to 1/10 of the control level) for mid-stage female pupae after treatment with this chemical at a concentration equivalent to *ca.* one-half of the LD_{50} dose, but not for male pupae.

Changes of Radiosensitivity to the Induction of Mutations in Primordial Germ-Cells During the Gonad Development of Female Silkworm

Mutsuo MIKI[1] and Akio MURAKAMI

The radiosensitivity to mutation induction in the female primordial germ-cells during the development of gonad in early stages of the silkworm embryos has been investigated. Eggs were collected from actively laying wild-type *C108* strain female moths crossed to the same strain of males at intervals of 30 min at 25°C. Developing embryos stages ranging from 44 to 212 hr after oviposition were irradiated at 12 or 24 hr intervals with a constant single dose of 1000 R of ^{137}Cs gamma-rays (dose-rate at 300 R/min). Irradiated eggs were reared and crossed after emergence, to normal males belonging to the marker stock homozygous for *pe* and *re*. The mutational incidence was used as the criterion for checking radiosensitivity.

The incidence of mutations increased gradually with the development of gonad in embryos from 68 to 116 hr after oviposition (or 24 hr before blastokinesis). After blastokinesis the incidence of mutations decreased

1) The Saitami-ken Sericultural Experiment Station, Kumagaya, Saitama-ken.

markedly in the 188 hr-old embryo to the level of the 44 hr-old embryo. The frequency of mutations in the 116 hr-old embryo showed to be the highest and that was newly twice as high as the frequency of mutations in the 68 hr-old embryo. The frequency of mutations again increased slightly but significantly in the 212 hr-old embryo. According to histological observation of changes of the number of germ-cells during development of gonad, it was observed that one division cycle in priomordial germ-cells was required for an approximately 30 hr in embryos ranging from 68 to 128 hr after oviposition. The completion of gonad fromation was observed at the 116 hr-old embryo and blastokinesis was occurred 140 hr after oviposition. However, after blastokinesis, the multiplication of primordial germcells ceased temporarily until the onset of gonial divisions in older embryos 2 days before hatching.

From these experimental results it can be concluded that changes in the radiosensitivity in priomordial germ-cells during gonad development is dependent on whether these stages of cell division or not as well as in other stage germ-cells of the silkworm.

Toxicity and Mutagenicity of Cadmium and Furylfuramide in Drosophila melanogaster

Yutaka INOUE and Takao K. WATANABE

Cadmium ($CdCl_2$) and furylfuramide (AF-2) were fed to *Drosophila* larvae throughly mixed with the standard medium of *Drosophila*. The toxicity of $CdCl_2$ has been detected by the concentrations of 50–75 ppm in fecundity, 75–100 ppm in viability and 1–10 ppm in developmental time. The toxicity of AF-2 has also been detected by the concentrations of 500–1,000 ppm both in fecundity and viability, and 0–200 ppm in deveopmental time. Thus, the developmental time is a most sensitive physiological trait to test the toxicity of these chemicals.

The mutagenicity of these chemicals has been tested by the attached-X method for the detection of mutations on the X chromosome. Statistically significant mutations could not be found in both cadmium (50 ppm) and AF-2 (200 ppm) treatments by this method. Recessive lethal or sterile mutations were accumulated in the second chromosome by the Cy-Pm method for many generations. Cadmium and AF-2 again did not increase the lethal and sterile mutation rates significantly. For details, see Japan. J. Genetics **53**, 183–189.

VII. RADIATION GENETICS AND CHEMICAL MUTAGENESIS IN MICRO-ORGANISMS AND PLANTS

Repair Enzymes for Gamma-Ray-Damaged DNA in Bacterial and Human Cells

Tadashi INOUE, Akiko YOKOIYAMA and Tsuneo KADA

We have recently developed a new assay system for the detection of enzymes functioning in the repair of gamma-ray-induced damages (Noguti, T. and T. Kada 1975, Biochim. Biophys. Acta **395**: 294; Inoue, T. and T. Kada 1977, Biochim. Biophys. Acta **478**: 234). In this system, the capacity of cellular extracts to enhance the priming activity of gamma-irradiated colicin E1 DNA for purified DNA polymerase I was measured. Using the system, we have successfully identified in extracts of *Bacillus subtilis* cells two kinds of enzymes (primer activating enzymes) which may function in the repair of gamma-irradiated DNA. One of them was shown to be an apurinic endonuclease and the other was a 'cleaning' exonuclease. Since gamma-rays induce various types of lesion in base and sugar moieties as well as scissions in the phosphodiester linkage, a wide variety of enzymes which function in repair may be detectable by this assay system, provided that the lesions are removed by excision-type repair passway in which DNA polymerase plays a significant role.

More recently we have applied the system to detect enzymatic defect of the human genetic disease ataxia telangiectasia which is characterized by telangiectasia, immunodeficiency, an increased frequency of malignancy and hypersensitivity to ionizing radiation. Preliminary studies revealed that an ataxia strain had substantially lower activity of the primer activating enzyme than a normal strain (Inoue, T. *et al.* 1977, Biochim. Biophys. Acta **479**: 497). Encouraged by these data, we examined nine ataxia strains and six normal strains in collaboration with Prof. B. A. Bridges and Drs. C. F. Arlett and A. R. Lehmann at the University of Sussex, as to the activity of the primer activating enzyme. The preliminary experiments yielded the results that all of the ataxia strains examined had lower activity than normal strains confirming that some, if not all, of the causes of the disease are deficiency in the repair enzyme activity for gamma-ray-

damaged DNA.

Rec-Assay with *Bacillus subtilis* Spores

Koichi HIRANO, Hisako MATSUMOTO and Tsuneo KADA

The rec-assay using *Bacillus subtilis* strains, M45 (Rec^-) and H17 (Rec^+) was tried with their spores on thin diffusion agar and remarkable improvements were made as to its sensitivity, quantitatively and reproducibility. The typical procedures are as follows. 10 ml of the molten broth agar (0.8% agar) containing about 2×10^5/ml spores of the strain H17 or M45 were poured at 42°C into an empty petri dish and solidified at room temperature. A paper disk impregnated with chemical solution was placed on the surface of broth agar and incubated at 37°C for 20 hours. Then the diameter of inhibition zone appeared around the disk was measured and compared for Rec^+ and Rec^- strains. For metabolic activation, the usual S9 fraction was prepared from PCB-treated rats and its 0.1–0.3 ml were mixed with broth agar. A cofactor solution containing 80 mg per ml of NADP and 40 mg per ml of Glucose-6-phosphate was prepared and its 20 μl were impregnated in the paper disk and diffused simultaneously with the drug (Details to be published as a full paper or in a chapter of "Chemical Mutagen" Vol. 6, Plenum).

Antimutagenic Action of Cobalt Chloride in *Escherichia coli* B/r WP2 *try*

Tsuneo KADA, Masako HARA and Nobutake KANEMATSU

We found that MNNG (N-methyl-N′-nitro-N-nitrosoguanidin)-induced mutations are remarkably reduced in *Escherichia coli* by the presence of cobalt chloride. The strain *E. coli* B/r WP2 was used and reversions to ptototrophy were studied. Broth-grown cells were washed with phosphate buffer and treated with MNNG and plated after appropriate dilution on broth-enriched (1%) minimal agar containing cobalt chloride ($CoCl_2\cdot6H_2O$) at different concentrations (0 to 25 μg/ml). In an experiment, the level of survivals after the mutagen treatment was about 80%. The presence of cobalt chloride in the agar medium reduced drustically the frequence of MNNG induced Try^+ revertants; 686.8 per 10^8 survivals in the absence of cobalt chloride, and 10.0 per 10^8 survivals in the presence of cobalt

chloride (20 μg/ml). Similar observation was made with ultraviolet light-induction of reversions in the same strain.

We are examing if the metal compound might block the mutagen-induced error-prone repair function in the cell (Proc. Japan Acad. **54**, Ser. B, 234, 1978).

Antimutator Effect of Cobaltous Chloride on a *Bacillus subtilis* Mutator Strain

Yoshiko OHTA, Tadashi INOUE and Tsuneo KADA

We have recently reported that cobaltous chloride ($CoCl_2$) reduced MNNG-induced mutation ($Trp^- \rightarrow Trp^+$) in *Escherichi coli* WP2 B/r (Kada, T. and N. Kanematsu 1978, Proc. Jpn. Acad. **54**(B): 234). We therefore examined whether $CoCl_2$ is effective on spontaneous mutation frequency in a *Bacillus subtilis* mutator strain. *B. subtilis* NIG 1125 (*met his mut*-1) is a strong mutator strain. The mutation *mut*-1 reduces DNA polymerase III activity, and elevates spontaneous mutation frequency (Brazill, G. W. and J. D. Gross 1973, Nature New Biol. **243**: 241). Two mutation, *met* and *his* possess the same suppressible nonesence codon (Okubo, S. and T. Yanagida 1968, J. Bacteriol. **95**: 1187).

Growing cells in Penassay broth were harvested and washed by centrifugation. Washed cells were plated on semienriched minimal agar plates containing various concentration of $CoCl_2$ and 20 μg/ml of L-methionine. On the above plates, mutation frequency ($His^- \rightarrow His^+$) was approximately 2.4×10^{-5}, whereas on plates containing 0.1 mM of $CoCl_2$, the frequency was found to reduce to 1.2×10^{-6}.

One explanation for the mode of action of $CoCl_2$ is that $CoCl_2$ inhibits the cell division which is neccessary to induce the mutation. In order to examine this possibility, the cells were plated on minimal agar which contained $CoCl_2$ and limited amount of nutrient broth. On these plates, cells can divide only a few times due to the limitation of the broth. After the incubation, cells were recovered from the plates and viable cells were scored to determine the number of cell division on the plates. Experiments with the plates containing various amount of the broth and $CoCl_2$ yeilded the results that $CoCl_2$ *did* reduce the mutation without the effect on the cell division.

Desmutagenic Activities in Vegetables and Fruits

Tsuneo KADA, Masako HARA and Kazuyoshi MORITA

Sorbic acid reacts with sodium nitrite and produces at least several rec-assay positive mutagenic nitroso- and nitro-compounds. Formation of many other mutagenic (and carcinogenic) nitroso-compounds is known in food. It may be fortunate if we have anti-mutagenic factors for the above mutagens in our food. We found by means of the rec-assay procedures, that vegetable or fruits such as pumpkin or cabbage contain factors which inactivate reaction products between sorbic acid and sodium nitrite.

Recently, it has been reported that smokes or pyrolysates of cigarettes, fish or meat contain mutagens of high activities, and the pyrolysis products of proteins and amino acids must share important parts of their total mutagenic capacity probably related to their carcinogenicity. Our recent observation revealed that juices prepared from cabbage, broccoli, green pepper, egg plant, apple, burdock, shallot, ginger, pineapple and mint leaf possess strong capacities of inactivating the mutagenicity of tryptophane pyrolysis products. In addition, radish, sweet potato, grape, Japanese ginger, cauliflower, beefsteak plant, enokidake mushroom and simeji mushroom were moderatly effective. Among the above eleven samples that inactivated the tryptophane pyrolysate efficiently, egg plant, burdock and broccoli showed the widest spectra of inactivating other mutagenic amino acids pyrolysates (Mutation Res. **53**, 351, 1978; Agrc. Biol. Chem. **42**, 1235, 1978).

Purification and Properties of a Desmutagenic Factor from Plant (*Brassica oleracea*) for Mutagenic Principle of Tryptophan Pyrolysate

Tadashi INOUE, Kazuyoshi MORITA and Tsuneo KADA

The *salmonella*/microsome mutagenicity test of Ames (Ames, B. N. *et al.* 1975, Mutat. Res. **31**: 347) has been widely employed for screening for chemical mutagens in our environment. By using the system, Sugimura and his collegues (Sugimura, T. *et al.* 1977, Proc. Jpn. Acad. **53**: 58) have recently found in pyrolysate of tryptophan an extremely potent mutagen, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) whose mutagenicity is comparable to that of aflatoxin B1 which is one of the most potent

carcinogen so far found. Since the pyrolysis product is very likely to be produced by cooking proteinous foods such as fish and meat, and since the correlation between mutagens and carcinogens have been generally accepted, one can speculate that such a mutagenic pyrolysis product plays some roles in carcinogenesis in human. It may therefore be very fortunate if we have desmutagenic factors for it in our environment especially in foods. With this view in mind we have examined some sixty species of vegetables as to the capacity of their extracts to inactivate the mutagenic principle and found that several kinds of vegetables including cabbage (*Brassica oleracea*) had considerable capacity to inactivate the principle (Kada, T. *et al.* 1978, Mutat. Res. **53**: 351; Morita, K. *et al.* 1978, Agric. Biol. Chem. **42**: 1235).

The desmutagenic factor was purified from extract of cabbage leaves by successive application to chromatographies on DEAE-cellulose, CM-cellulose, Sephacryl S200, DEAE-Sephadex and CM-Sephadex. The final preparation was approximately 360 times purified; and upon SDS-gel electrophoresis, only one protein band was present and its position of migration corresponded to a molecular weight of about 43,000. The purified factor exhibits the absorption spectrum with maxima at 278, 404 and 497 nm and small shoulder at around 380 nm. The absorptions at the wave length maxima are present in the following ratio: 0.332, 1.000 and 0.089, respectively; The extinction coefficient $E^{0.1\%}_{404nm}$ was calculated to be 7.33. These spectral properties suggest that the desmutagenic factor contains a prosthetic group of a heme-like chromophore. We therefore examined the effect of reducing agent on the spectrum. Treatment of the factor with sodium hydrosulfite resulted in partial bleaching of the 404 nm band and in shift the band to 436.5 nm. This result strongly indicates that the factor is a hemo-protein. Analysis of iron by the atomic absorption revealed that the factor contained 0.624 μg of iron per mg of protein. If we assume that the molecular weight of the factor is 43,000 as determined by SDS-gel electrophoresis, it was calculated that 0.492 mole of iron per 1 mole of the protein. In addition to Trp-P-2, the mutagenicity of Trp-P-1, which is 4-methyl derivative of Trp-P-2 and is also contained in pyrolysate of tryptophan, was effectively abolished by the factor. Ethydium bromide and 2-aminoanthracene were also susceptible to the factor. However, the factor had no effect on the mutagenicity of ICR-170 and AF-2. The mutagenicity of 1,2-diamino-4-nitrobenzene was greatly

enhanced by the factor.

The mode of action of the factor on the mutagens is under investigation.

Dose Response of Maize to Chronic Gamma-Ray Irradiation*

Etsuo AMANO and Yasuo UKAI**

To study the mutagenic effects of low-dose rate chronic gamma-ray, maize (*Zea mays*) were planted in Gamma Field of Institute of Radiation Breeding, NIAS, Ohmiya-machi, Ibaraki-ken. For examination of size of mutant tissues in chronically irradiated plants, yg_2/Yg_2 heterozygotes were used, and for the response in lower dose rate range, *wx*/*wx* inbred line was used to score possible mutants in pollen grains.

A mutant cell induced by irradiation would proliferate and develop to form a mutant tissue. The size of this mutant tissue might vary depending on the growth after the induction of the mutation. If the size, or the number of cells involved, vary too much in chronically irradiated materials, reliability of mutant pollen scoring would be reduced, for the pollen grains were pooled in the scoring disregarding the position in the inflorescences. To examine this, yg_2/Yg_2 heterozygotes were planted in the Gamma Field and compared with the semiacute irradiation. Semi-acute irradiation was made by irradiating 20 days old seedlings in the same Gamma Field for five days, but with approximately 5 times higher dose rate upto 473 R/day. For chronic irradiation, materials were planted in the Gamma Field at the same age, and irradiated until harvest of leaves, dose rate ranging from 15.2 R/day to 83.0 R/day. The final leaves were collected to examine the mutant sectors. Lengths of the light colored sectors were measured and their relative locations on the leaf were recorded. The average size of the mutant sectors of the semi-acutely irradiated materials were larger than the chronically irradiated ones, and many of the sectors extended from their initiation points to nearly tip of the leaf. Compared to this, most of the light colored sectors on leaves of the plants which were chronically irradiated all through the growing stage were small. Only a few percent of the sectors exceeded five per cent of the leaf length. In scoring of the sectors, only the sectors which exceeded 1 mm were adopted, but many smaller sectors were also noticed. This might indicate that practically the size of

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** Institute of Radiation Breeding.

the mutant tissue were so small and homogeneous that mutant pollen scoring might reflect the mutant frequency of the materials. Moreover the mutatnt sector frequencies in the irradiated materials were 20 times more in chronic irradiation.

Mutant pollen frequencies were examined in chronically irradiated *wx/wx* inbred line (No. 639). Pollen grains can be easily collected and examined up to 10^6. The waxy character can be visualized by iodine staining. Normal non waxy (*Wx*) pollen grain stains blue black. Waxy mutant (*wx*) shows reddish brown color. But the color change by forward mutation from *Wx* to *wx* may not be separable from physiological damage of the pollen, i.e. the lightening of the stained color. The use of the *wx/wx* enabled the detection of the second starch character gene, the amylose extender (*ae*). The materials used were *wx*, *Ae/wx*, *Ae*. Mutation from *Ae* to *ae* increases amylose like component in starch changing the color of the iodine stain darker. Reverse mutation from *wx* to *Wx* would be rare, but it would be detected likewise.

Male tassels were collected and fixed in 70% alcohol before anthesis. About 40 flolets per slide were taken out and homogenized in Nelson's iodine solution (0.18% I_2 in KI solution). The suspension was filtered through cheese cloth on the large slide glass. The number of the total pollen grains were estimated by scoring the pollen at 15 places. Dark stained mutant pollen grains were scored by scanning all the prefixed $50\times$ 75 mm^2 area. Distribution of the mutant frequency along the main axis of the tassel did not deviate very much, again supporting the results of the leaf sector scoring. The mutant frequency increased almost linearly up to 10 R/day and 1×10^{-2} mutant frequency, in parallel with the pollen sterility. The lowest dose rate that increased the mutant frequency were 3.8 R/day at 92 m from the source. It was twice in the distance and 1/4 in intensity for yg_2 leaf sector scoring. The results indicated that pollen grain scoring was a very sensitive mean to study the mutagenic effect of low dose rate gamma-ray. Preliminary results showed linear increase of mutant frequency with the dose rate.

Measurement of Waxyness in the Induced *wx* Mutants

Etsuo AMANO

Ethyl methanesulfonate (EMS) is a very effective chemical mutagen. It

may induce base pair substitution in higher plants. This was shown by presence of CRM in sh_1 mutants in maize by Chorey and Schwartz (1971, Mut. Res. **12**) in maize. Frequently leaky mutants which would be indications of missence mutation, were found in *C* locus, *wx* locus in maize (*Zea mays*) and *wx* mutants in rice plant (*Oryza sativa*). Measurements of this leakyness were tried by modified blue value method for the iodine stained starch solution. The measuring device consisted of stabilized incandescent lamp, thermal filter, sample cell, beam divider mirrors and two silicon photo-cells with 430 nm or 660 nm interference filter. A 10 mm × 10 mm sample cell was deviced with a small mixing motor and inlet and outlet tubes to enable continuous dilution of the sample solution. Ordinally blue value method to measure the amylose, the *Wx* component, which stains blue by iodine, use the absorption of 660 nm red light of the solution of certain concentration of total starch. The present measurement used the 430 nm blue light to determine the concentration of the starch solution. DC amplifier and digital printer recorded the transmission value of 660 nm at the instant when the iodine stained solution in the sample cell was diluted to the concentration of 50% transmission for 430 nm.

Each cereal grains were crashed and put into test tubes. Distilled water was added to the tubes, 3 ml each for rice grains and 5 ml each for maize grains. Tubes were autoclaved at 1 atm. for 15 min. Tubes were stirred while warm. After cooling down to room temperature, 1 ml supernatant solution was taken and mixed with 0.1 ml of 0.09% I_2-KI solition (1/2 of Nelson's I_2-KI solution) in the sample cell. Then measured the transmission of 660 nm light at the 50% transmission concentration for 430 nm light. The T-value in percent for 660 nm was used as index to show the waxyness. The measuring system was as sensitive as to take a single rice grain as a sample, thus distribution of the *wx* index among a population could be analyzed. The index value of normal *Wx* starch was about 20 in rice and 17 in maize. The standard waxy mutants showed the indices, around 70 in rice and 65 in maize. In maize, five out of eight EMS induced mutants examined showed nearly equal value to small deletion mutant wx^R. The rest of the mutants showed lower index values. In rice, four out of eight EMS induced mutants showed the indices as high as the standard *wx* mutant. Other four mutants showed lower index numbers.

Examinations of self pollinated F_2 populations in maize showed clear 3: 1 segregation indicating complete dominance of *Wx* over *wx*, regardless

of the *wx* mutant lines examined. Compared to this, in rice, segregations were not so clear as in maize. Recessive *wx* group could be separated, but *Wx* group distributed from standard *Wx* to almost half way to *wx*. The frequency spectra indicated 2: 1: 1 distribution suggesting gene dosage effect in the triploid endosperm. The numbers would correspond to *WxWxWx* and *WxWxwx*, *Wxwxwx*, and *wxwxwx*. This gene dosage effect had been noticed visually in endosperm, and the progeny tests supported the explanation by gene dosage effect.

Modification of Radiation Damage and Variation of Mutant Sector in Maize with Different Kinds of Radiations

Taro FUJII

Mutation from dominant *Bz* to recessive *bz* was detected as a phenotypic change in the aleuron color of M_1 seeds in the cross of a recessive stock with the irradiated pollen grains carrying the dominant gene. Large variations in the mutated area of M_1 seed surface was noticed, viz. whole area covered with mutated character—whole type, and mutated character appeared in a limited part of seed surface—partial type. Furthermore, partial type shows wide variation in its sector size. To investigate the correlation between mutation type and radiation quality, partial type was divided into four categories; nearly-whole—almost all area of seed surface covered with mutated character, 1/2—about 50% is mutated area, 1/4—about 25% is mutated area, and dot—mutated character appeared as a

Table 1. Mutation detected and radiation type
Frequency distribution in

	Whole: Partial		Deline in mutation frequency	the partial type (%)			
				Neary whole	1/2	1/4	Dot
Gamma-rays	2	1	—	50	20	20	10
〃 (Fractionation)	1.5	1	Yes (only in whole)	50	20	20	10
〃 (Low dose rate)	2	1	Yes	50	20	20	10
14 MeV neutrons	2	1	—	50	20	20	10
〃 (Fractionation)	2	1	No	50	20	20	10
Ultraviolet	1	4–5	—	20	50	20	10
〃 (Photoreactivation)	1	4–5	Yes	20	50	20	10

dot, and frequency distribution of these categories was examined. Summary of the results are shown in Table 1. Dose range used in the present study is 500–3000 R of gamma-rays, 250–680 rad of neutrons and 4000–12000 ergs/mm^2 of ultraviolet. No dosage effect was observed in each type of radiation on the ratio of whole and partial types, as well as in the frequency distribution among the partial type.

Recovery of premutational damage was observed with fractionation treatment merely in whole type in the gamma-ray induced mutations (40 kR/h intensity). However, at low dose rate (1 kR/h) mutation frequency declined both in whole and partial types. On the other hand, mutation frequency did not show any significant difference with single and fractionated treatments of neutrons. Nevertheless, whole and partial mutants were always in the ratio of 2: 1 in ionizing radiations, except gamma-ray fractionation treatment. The same frequency distribution in the partial type was observed both in gamma-rays and neutrons.

In the ultraviolet induced mutations, ratio of the partial type is very high, frequency distribution in the partial type is also quite different from those of ionizing radiations. Although the decline in mutation frequency is very clear with photoreactivation treatment both in whole and partial types, change in the frequency distribution in the partial type was not observed either with or without photoreactivation treatment. Thus, the nature of damage caused by UV-irradiations which differ greatly from that of ionizing radiations may reflect on the mutation type. Mechanism responsible for these mutation types are still obscure, and dual mechanism, lethal hit, master strand or miss repair hypothesis have been proposed to explain the mechanism of their origin. However, ratio of whole and partial, and frequency distribution in different categories of partial mutations shows a constant ratio according to the kind of radiation.

VIII. POPULATION GENETICS (THEORETICAL)

Theoretical Study of Genetic Variability, Assuming Stepwise Production of Neutral and Very Slightly Deleterious Mutations

Takeo MARUYAMA and Motoo KIMURA

Mathematical treatments are presented that enable us to compute the amount of genetic variability maintained in a finite population, assuming that mutations occur in stepwise fashion and that both selectively neutral and slightly deleterious alleles are involved. Two numerical examples show that, if very slightly deleterious mutations are prevalent, the amount of genetic variability increases much more slowly as the population number increases than is the case when all the mutations are strictly neutral. (For details see Proc. Nat. Acad. Sci. USA **75**: 919–922, 1978).

Extension to the Neutral Mutation Random Drift Hypothesis

Tomoko OHTA

Effects of varying selective force (not fluctuating from time to time for a single mutant but varying over many newly arising mutants) on the average heterozygosity and the evolutionary rate have been investigated under the hypothesis assuming prevalence of very slightly deleterious mutations. It has been shown that, as the population size increases, the rate of approach to the upper limit of heterozygosity under negative selection is slower for the case of varying selection coefficients as compared with the case of constant selection. Also, an inverse relationship between the evolutionary rate and the population size is shown to hold if the selection coefficients against mutant alleles follow an exponential distribution.

The theoretical distribution of genetic distance was studied using Monte Carlo experiments assuming selective neutrality. It agrees reasonably well with the observed distribution in *D. willistoni* group. However, under the more restricted condition of measuring the distance for polymorphic cases only, there is a discrepancy in the distribution of distances between the actual observations and the theoretical expectations. It was pointed out that the hypothesis of very slightly deleterious mutations (a modified

neutral theory) offers a most natural interpretation for this kind of minor discrepancy. Details were published in Proc. of The Second Taniguchi International Symp. on Biophysics, "Molecular Evolution and Polymorphism" (ed. M. Kimura), pp. 148–167.

On the Gene Conversion Model as Mechanism for Maintenance of Homogeneity in Systems with Multiple Genomes

Tomoko OHTA

The gene conversion model reported by Birky & Skavaril (Genet. Res. **27**, 249, (1976)) has been analytically studied by using the theory of diffusion models of Kimura (Jour. Applied Probability **1**, 177, (1964)) in population genetics. It has been shown that the fate of new mutations in systems with multiple genomes may be satisfactorily treated by the diffusion model. For details, see Genet. Res. **30**, 89–91.

IX. POPULATION GENETICS (EXPERIMENTAL)

Number of Sterile Loci in *Drosophila melanogaster*

Takao K. WATANABE and Won Ho LEE

The number of loci which are potentially able to produce sterility genes was estimated for *D. melanogaster*. The frequencies of induced and spontaneous sterility mutations and that of simultaneously occurred lethal mutations were compared, together with some data from published literature. The mutagenic strength of the chemical treatment differed to some extent between experiments, and treated chromosomes. However, if mutations to lethality and sterility are compared within each experiment and presented as relative mutation ratios, these values are useful for estimating the standard mutation rate of sterility genes.

To standardize the mutagenic effects of various mutagens on various chromosomes, the frequency of male-sterile (dm) or female-sterile chromosomes (df) was divided by the frequency of lethal chromosomes (l) in each experiment. Although the ratios dm/l or df/l were varialbe among experiments, they were apparently smaller than 1. A maximum likelihood estimate was 0.153 for dm/l and 0.115 for df/l.

If we suppose that sterile and lethal mutations per locus occur approximately at the same rate, the number (Ns) of loci which are able to have sterile can be calculated, $Ns=Nl\times(d/l)$. Since the number of lethal loci (Nl) on the second chromosome is about 500 (Wallace 1950 PNAS **36**: 654), the number of potentially male-sterile loci is $500\times0.153=76.5$ and that of female-sterile loci $500\times0.115=57.5$ on the second chromosome. For details, see Genet. Res. **30**, 107–113.

Evolutionary Changes of Inversion Frequencies in a Natural Population of *Drosophila melanogaster*

Yutaka INOUE and TAKAO K. WATANABE

Inversion polymorphism in natural population of Katsunuma has been studied for more than ten years. The frequency of polymorphic inversions was relatively high at sixties where the average frequency was about 25%,

whereas it decreased at seventies to be about 15%. Simultaneous reductions of several kinds of inversions suggest that natural selection was acting against such inversions (*2Lt, 2RNS, 3LP, 3RM*$_0$) in similar way and it became stronger in these years.

On the other hand, an inversion (*3RP*) has survived for these ten years without any frequency change. Two new inversions, *2LW* (28C; 32C) and *3LY* (68F; 75C), have recently appeared and settled by the frequency of about 3% in the population. These three inversions must have different adaptive strategies from the above inversions, since they have survived rather constantly or more adaptively in these years.

The reduction of some inversion frequencies in Katsunuma has changed the order of relative abundance of four polymorphic inversions in four major autosome-arms. The order was *2Lt*>*2RNS*>*3RP*>*3LP* in the sixties, but it became *3RP*>*2RNS*≧*2Lt*>*3LP* in the seventies. The *2Lt* decreased so rapid that its frequency became close to or somewhat less than the frequency of *2RNS* whose decreasing rate was relatively slow.

X. EVOLUTIONARY GENETICS

Preponderance of Synonymous Changes as Evidence for the Neutral Theory of Molecular Evolution

Motoo KIMURA

According to the neutral mutation-random drift hypothesis of molecular evolution and polymorphism proposed by the author in 1968 (Kimura, Nature **217**, 624), most mutant substitutions detected through comparative studies of homologous proteins (and the nucleotide sequences) are the results of random fixation of selectively neutral or nearly neutral mutations. This is in sharp contrast to the orthodox neo-Darwinian view that practically all mutant substitutions occurring within species in the course of evolution are caused by positive Darwinian selection.

Following the neutral theory, we consider two types of mutational changes, neutral and deleterious, and assume that the probability of a mutation being neutral (not harmful) depends on the functional constraint of the molecule. Namely, the stronger the constraint the smaller the probability of selective neutrality.

It is known that a large fraction (roughly 2/3) of nucleotide substitutions at the third positions of the codon are synonymous, that is, they do not cause amino acid changes in proteins. Since natural selection acts through phenotypes, and particularly, through the function of the protein, it is expected that synonymous changes are subject to much weaker natural selection, if any, than nucleotide changes causing amino acid changes. This means that, if the neutral theory is valid, the rate of mutant substitutions at the third positions of the codon must be very high.

Recently, it has become possible to estimate the evolutionary rate of synonymous mutant substitutions by using data obtained from comparative studies of messenger RNA sequences.

Salser *et al.* (*Fedn. Proc.* **35**(1), 23 (1976)) have presented a comparison of homologous parts from the fragments of the human and rabbit hemoglobin mRNA sequences. Using their data, it is estimated the rate of nucleotide substitutions per site per year at the third position of the codon is

$$k_{\mathrm{unc}}=(2.3\pm1.1)\times10^{-9}.$$

This is a very high evolutionary rate comparable with that of the fibrinopeptides.

A similar but more interesting report comes from Grunstein *et al.* (J.M.B. **104**, 351 (1976)) who compared histone IV mRNA sequences of two sea urchin species. It is well known that histone IV is by far the most highly conserved protein with the rate of amino acid substitution $k_{aa}=0.006\times10^{-9}$ per year. Their report now shows, however, that many synonymous changes have occurred in the gene coding for this protein in the course of evolution. Using Grunstein *et al.*'s data, the following estimate has been obtained for the rate of nucleotide substitutions at the third nucleotide position of the codon in the histone IV genes.

$$k_{nuc}=(3.7\pm1.4)\times10^{-9}.$$

These estimates conform remarkably well with the framework of the neutral theory. Let k be the rate by which mutant genes are substituted in the species in the course of evolution, and let f_0 be the fraction of molecular mutants that are selectively neutral. Since, for neutral mutants, the rate of mutant substitution is equal to the mutation rate, we have

$$k=v_T f_0$$

where v_T is the total mutation rate. According to the neutral theory the probability (f_0) of a random change being selectively neutral is larger the weaker the functional constraint with the result that k in the above equation gets larger. It is clear that the maximum evolutionary rate is attained when $f_0=1$, that is, when all the mutations are neutral. Now, the high evolutionary rates observed at the third position of the codon can be explained from the neutral theory by assuming that the majority of synonymous changes are selectively neutral (Note that roughly 2/3 of random nucleotide substitutions at the third position of the codon are synonymous). On the other hand, if we adhere to the selectionist position that practically all the mutant substitutions in evolution are caused by positive natural selection, there can be no upper limit to the evolutionary rate at the molecular level (as directly set by the mutation rate v_T).

In my opinion, various observations suggest that as the functional constraint diminishes the rate of evolution converges to that of the synonymous substitutions. If this turns out to be true, such a convergence or plateauing of molecular evolutionary rates will turn out to be strong supporting

evidence for the neutral theory. For more details, see Nature **267**, 275–276 (1977).

A New Hypothesis for the Direction of Evolution among Related Species of *Drosophila*

Takao K. WATANABE and Masaoki KAWANISHI

Drosophila females usually choose their mating partners if males of different species are present, while males generally show no mate preference. The degree of successful matings between pairs of reciprocal hybrid matings often differs and this indicates the direction of evolution among the sbling species.

For example, *D. melanogaster* females were always more successful in mating with *D. simulans* and *D. mauritiana* males than the reciprocals. On the other hand, *simulans* females were more successful in mating with *mauritiana* males than the reciprocal cross, but were less successful with *melanogaster* males than the reciprocal. Finally, *mauritiana* females were always less successful in mating with both *melanogaster* and *simulans* males than were the reciprocal crosses. If we hypothesize that females of a newly evolved species tend to reject males of the original (ancestral) species, the direction of evolution would be *melanogaster*→*simulans*→*mauritiana*.

The direction of evolution in the *virilis* group of *Drosophila* has been discussed on the basis of cytological (Stone *et al.* 1960, PNAS **46**: 350), morphological (Throcknorton 1962, UTP 6205: 207), and electrophoretical (Hubby and Throckmorton 1965, Genetics **52**: 203) studies. Investigations of these different features have resulted in the conclusion that *virilis* is the original species and th other species have been derived as follows: *virilis*→*novamexicana*→*americana*→*texana*, and *virilis*→*lacicola* and *montana*. This relation can be perfectly substantiated by the present hypothesis by using data of Patterson *et al.* (1947 UTP 4720: 7, 1949 UTP 4920: 7). That is, females of a derived species discriminate and reject males of the ancestral species whereas females of the ancestral species readily accept males of the derived species.

Mating preference and cytological data indicate the same direction of evolution in the *mulleri* group of *Drosophila* (Wasserman 1963, Am. Nat. **97**: 333; Patterson 1947, UTP 4720: 32). The phylogeny of *D. willistoni*

group established from the electrophoretical differences (Ayala *et al.* 1974, Evolution **28**: 576) was almost exactly substantiated by our method when it was applied to the data (Buria *et al.* 1949, Evolution **3**: 300; Koref-Santibanez 1972, Evolution **26**: 326).

Our hypothesis is based on the assumption that a newly derived species would have gained some cortship pattern in addition to the ancestral one. Because of this the derived females show strong discrimination against males of ancestral species which lack the full courtship pattern, but derived males are still accepted by the ancestral females which require a partial (ancestral) courtship pattern.

XI. HUMAN GENETICS

Reexamination of Paternal Age Effect in Down's Syndrome

Ei MATSUNAGA

Paternal age distribution for 1279 cases of Down's syndrome born in 1952–1968 was compared with the corresponding distribution for the general population, taken from the vital statistics data and corrected for the maternal age as well as for the year of birth of the patients. Although there was no difference in the mean paternal age, the two distributions differed significantly, largely due to the excess of fathers aged 55 years and over and to the deficit of those aged 40–44 years in the patients born to mothers aged 30 years and over. The overall pattern of the relative incidence of Down's syndrome with advancing paternal age, with maternal age controlled, seems to be consistent with the hypothesis proposed by Stene *et al.* (1977 Ann. Hum. Genet. **40**: 299); it increased from 0.8 for fathers aged 20–24 years slowly up to 1.2 for those aged 45–49 years, though with an intermediate drop to 0.8 at the age of 40–44 years, and then sharply to 2.4 for those aged 55 years and over. This rising pattern of the relative incidence with paternal age was essentially the same for the patients born in 1952–1960 and for those born in 1961–1968, although the slope was less steep in the latter than in the former group. In addition, for those patients born to younger mothers (under 30), the relative incidence increased lineally up to the paternal age of 39. For details, see Hum. Genet. **40**: 259–268 (1978).

A Genetic and Epidemiologic Study of Polydactyly in Human Embryos

Kohei SHIOTA and Ei MATSUNAGA

Among the large collection of human conceptuses in the Human Embryo Center for Teratological Studies, Kyoto University, 129 embryos with polydactyly were found in the period from 1962 to 1974. The overall incidence of this anomaly in human embryos was 0.35%, which must be an underestimate because the denominator includes a large number of damaged embryos. A significant increase was noted in 1971 and 1972, and some exogenous factors, most probably ascertainment bias, were sus-

pected to be responsible. About three quarters of the present cases had preaxial polydactyly in contrast to the predominance of postaxial type in Negro and Caucasian populations. The ratio unilateral to bilateral affection was approximately 2: 1. The mean maternal age did not differ significantly from that of the general embryonic population. Maternal pregnancy order had no effect upon the causation of this malformation. The case mothers were shown to have more frequent miscarriages than controls, suggesting the possible effect of some kind of maternal predisposition. No association was verified with paternal age, parental consanguinity nor with maternal medical history, including irregular menstrual cycles, and smoking and drinking habit. None of the cases was found to be familial. Dominant inheritance does not seem a major cause and complex genetic mechanisms or unknown exogenous factors may be responsible for a majority of the Japanese cases of polydactyly. Details have been published in Jap. J. Human Genet. 23: 173–192 (1978).

Palindromic Theory and the Telomere of Eukaryote Chromosomes

Yasuo NAKAGOME

The known DNA polymerases act in the 5′ to 3′ direction and require a free 3′-OH as a primer. Thus the mechanism of replication of the 5′ end of a linear DNA molecule remains unclear. A few models, including palindrome models of Cavalier-Smith and Bateman, have been proposed (Nature, **250**: 467, 1974 and **253**: 379, 1975).

In eukaryote chromosomes, a telomere behaves differently from a broken end in that it has no tendency to join with the latter. Cavalier-Smith pointed out that the existence of "terminal palindrome could explain several intriguing properties of the telomeres." If telomeres have a structure proposed by Bateman, 50% of them consist of DNA with two newly syntheiszed strands and the rest with two old strands (see Fig. 1 of Bateman). If the Cavalier-Smith's model is the case, each telomere consists of each one of old and new strand. In only the Bateman model, a telomere with two old strands can arise.

In the present study, human diploid cells (WI-38) were incubated in the presence of BUdR for the length of $S+G_2$ phase. Metaphases were stained with acridine orange. The experiment was designed to detect intense fluorescence of regions with two old strands. The technique was

essentially the same as that used in our previous work (Nakagome, Exp. Cell Res., **106**: 457, 1977). In most metaphases, chromosomes showed dull and homogeneous appearance and none of more than 1000 telomeres fluoresced intensely. In some of metaphases, chromosomes showed R-band pattern, presumably representing cells with long generation time.

It was reported that single T2 phage was detectable by the use of acridine orange (Mayor and Hill, 1961). The virus has a linear DNA with 18.2×10^4 nucleotide pairs which exceeds the largest palindrome (2×10^4) isolated so far from eukaryotes by biochemical means. However, it does not preclude the possibility that telomeres contain longer palindromic sequence. There are only a total of 92 telomeres in an interphase nucleus and a specific sequence, if any, is not likely to be detected by biochemical means. It may be that telomeres of human chromosomes either have no DNA with two old strands (Bateman structure) or have one which is too small to be detected.

XII. BEHAVIORAL GENETICS

Circadian Locomotor Activity of Sensitive and Unsensitive Strains of *Drosophila melanogaster* for Noise Environment

Chozo OSHIMA and Won Ho LEE

The circadian locomotor activity of an individual male fly in a small glass chamber ($4 \times 10 \times 50$ mm) was recorded continuously by the electronic actograph. The record was usually taken for a week; the former half period was under light and dark daily cycle (L D) with dusk and dawn, and the latter half period was under constant dark (D D) and temperature was kept constant 25°C. The locomotor activity of flies of about ten homozygous strains for the second chromosome was observed to be daily rhythmic and showed two high peaks at dusk and dawn under L D environment. On the other hand, the free running locomotor activity of flies continued at low lebel during the subjective light period under D D environment. Under both L D and D D environments, the locomotor activity of flies stoped usually for about 6 or 7 hours of common dark period. Then, the pure sound (2000 cycle, 100 phon) was projected for 5 hours of the rest time.

Male flies of 3 homozygous strains 168, 145 and 146 felt the noise and moved not only for 5 hours, but also their excitement continued after several hours. On the contrary, male flies of 3 homozygous strains 16, 49 and 83 felt it a bit or not at all. The former strains were assumed to be sensitive and the latter ones were unsensitive (resistant) for the noise environment.

The most sensitive strain 168 and unsensitive strain 83 were crossed reciprocally. The locomotor activity of hybrid male flies was recorded. Their sensitivities for noise were middle between both parental strains, but close to the unsensitive one and their characteristics of locomotor activity rhythm at dusk and dawn under L D environment were similar to the sensitive one. From the result, the sensitivity for noise environment is presumed to be a recessive character.

Genetic Variation of Flying Ability of *Drosophila melanogaster*

Won Ho LEE and Takao K. WATANABE

The flying ability of *Drosophila* flies was measured by a glass cylinder apparatus, similar to Benzer's flight-tester (Scientific Am. **229**: 24, 1973). The inside wall of the cylinder was coated with liquid paraffin. Thirty flies of one sex, 2 or 3 days old, were gently aspirated into the apparatus. If flies can fly they strike on the wall from upper region (5) to lower region (2) according to their flying ability and fix there, and if they can not they fall to the bottom (1).

A total of 52 second chromosomes was isolated from Katsunuma of 1975. Homozygotes for each chromosome line were examined in the apparatus. Variations of flying ability in the homozygotes ranged from 2.6 to 4.4 with an average value of 3.88. Two lines showed very weak flying ability but no flightless lines were detected. Analyses of variance was significant among 52 lines, suggesting the flying ability was genetically controlled.

Correlations between the flying ability and other genetic traits such as walking ability, viability, body weight and wing length were not at all statistically significant. Thus, genes controlling flying ability were in large part different from those controlling other genetic traits examined above.

Effects of Noise on the Learning Performance of Mouse Strains with Different Learning Abilities

Tohru FUJISHIMA

The effects of noise on the learning performance of mice was examined under the following experimental conditions. Two strains, C3H/HeMs and SWM/Ms, were exposured to one-hour pink noises of 100 phon six times at one hour intervals every night for a period of one or three weeks, and they were tested with an automated Y-maze apparatus, using buzzer and lamp as the conditioned stimuli and electric shock as the unconditioned stimulus. In a previous experiment, the C3H showed the best discriminatory ability, while the SWM showed the best avoidance ability among several inbred strains tested (cf. Ann. Rep. **25**: 82).

Both of the two strains showed no remarkable depression in learning performance in the first training session after one-week or three-week

exposures to the noise environment. However, the two strains differed in their responses to noise. In the second training session, the SWM showed a slight depression in avoidance performance, while the C3H showed a little improvement in this performance. The learning test following an additional one-week exposure to noise, however, indicated significant depressions of performance in both strains, especially in avoidance performance. It was also found that noise made the mice emotional or nervous. They showed no acclimatization to the noise environment with regard to activity and learning performance.

Genetic Analysis of a Mutant Showing Abnormal Behavior in the Silkworm

Akio MURAKAMI and Akio OHNUMA

For a better understanding of animal behaviors, it is favourable to analyze mutants which show abnormal behavior in organisms with a simple nervous system. With this in view, observations were made to detect mutants in the silkworm, *Bombyx mori*. We report here the occurrence of a sex-linked recessive mutation showing abnormal behaviors regarding to muscle response in the larvae and sexual response in adults of *Bombyx*.

In this mutant line intersegmental membranes in all segments of larvae, specially in the aged larvae, are abnormally soft and pliant when compared with the normal stocks. Accordingly, the inability of the larva to keep a normal carrieage may be due to the disorder of neuromyal junction. It is also interesting to note that the mutant male moths can not respond to a sex-attractant of pheromone which is secreted from an alluring gland of female moths, although they are able to flutter when these males are hung with fingres. This suggests that this line male moth has a normal wing muscular system. It is further observed in this line infecundity and sterility caused by the abnormal sex-behavior pattern—its inability to mate. In spite of those abnormalities, this mutant silkworm develops normally without any significant reduction of viability. These evidences clearly suggest that the mutational trait may primarily affects the central nervous system or brain controlling behaviors in general rather than a special ganglion affecting partial behavior.

The abnormal syndrome was detected at first in only female offsprings from a cross between a normal female and an F_1 male in which the female

had been X-irradiated with a 1,000 R, the males did not show abnormal syndrome. However, subsequent sib-matings of those P_1 offspring produced larvae in the ratio 1 normal: 1 abnormal regardless of sex, suggesting that this syndrome is controlled by a single sex-linked recessive factor (or gene) *spli* (soft and pliable). To verify this point, females hemizygous for *spli* were crossed with heterozygous males (*spli*/+), the offsprings were wild-type and abnormally soft and pliable larvae in the ratio of 1: 1 regardless of sex. When wild-type female (+/+) were crossed with homozygous males (*spli*/*spli*), abnormally soft and pliable female larvae and wild-type male larvae were obtained. In addition, matings of abnormal females hemizygous for *spli* and wild-type males (+/+) produced only wild-type larvae for either male or female. Homozygous male larvae for *spli* have shown a typical syndrome the muscular dystrophy. It can concluded from these experimental results and others that this syndrome is under the control of a single recessive gene, *spli*, which is located on the X-chromosome. Preliminary investigations indicate that the *spli* is located between known gene loci *sch* (sex-linked chocolate: 21.5) and *e* (elongate: 36.4) on the X-chromosome. However, this has to be confirmed further.

Histological and electrophysiological analysis for this mutant line are in progress.

XIII. ECOLOGICAL GENETICS

Observations of Rice Species and Accompanying Savanna Plants at the Southern Margin of Sahara Desert

H. I. OKA, H. MORISHIMA, Y. SANO, and T. KOIZUMI*

In the early dry season (October-November) of 1977, we have travelled in the interior of Nigeria and other West African countries for ecological-genetic studies of rice species and accompanying savanna plants. Our trip was planned and realized under a grant for overseas field research of the Ministry of Education, and with the generous cooperation of the International Institute of Tropical Agriculture (IITA, Nigeria), Office de la Recherches Scientifique et Technique Outermer (ORSTOM, Chad and Ivory Coast), Institut de Recherches Agronomiques Tropicales (IRAT, Ivory Coast), and other agricultural institutions. Our objectives were to make observations on the diversity of plants as influenced by natural envrionment and human activity, and to collect the seeds of wild and cultivated rice species.

The seed samples collected were: *Oryza sativa*, 36; *O. glaberrima*, 31; *O. perennis* subsp. *barthii* (=*O. longistaminata*), 27; *O. breviligulata*, 20, and *O. punctata*, 8. Each accession consisted of seed samples taken at random from 20 to 30 plants of a pouplation, either on a single plant basis or in bulk. The seeds were shared with the units in charge of germplasm conservation of the IITA and ORSTOM.

Observations were recorded at 77 selected sites, of which 28 were swampy depressions (where wild rice species grew), 23 were upland savannas, and 26 were rice fields. Records were taken at each site with regard to physiography, water condition, degree of disturbance by man, grazing by animal, height and cover of major plant species, habitat segregation, and within-population variations in visually distinguishable characters. For rice fields, the method of cultivation was recorded in addition. On the basis of herbarium specimens collected at these sites, a total of 117 species were identified by Dr. I. O. Akobundu of the IITA. Further, for a survey of buried seed populations, soil samples were taken from 27 sites. Although

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experiments with the collected materials are under way, the following facts were pointed out from our field notes.

a) Savanna vegetation: Latitude is an important factor in determining natural vegetation as well as cropping system, as the higher the latitude, the shorter the rainy season and the more uncertain the rainfall. The proportion of annual species at a site tended to be high at higher latitude. Total biomass estimate (plant height×cover) tended to be small at higher latitude. Generally, species diversity was correlated with total biomass, though it differed according to topographic conditions and habitat disturbance.

b) Wild rice species: *Oryza perennis* subsp. *barthii* had rhizomes in all 27 populations observed. The populations were homogeneous in outward appearance except for a difference in awn color (red *vs.* white), even though they are known to have a large amount of enzymatic variations. In many places, the plants formed clumps as the result of their rhizomatous propagation. Some of the plants showed pollen and/or seed sterility.

The annual species, *O. breviligulata*, was found in swampy depressions in the Sudan and Sahel zones. In the area where the plants form big populations as in Chad and northern Cameroun, the people collect the grain as their food. Scattered plants of this species exhibiting some cultivated characters (resembling *O. glaberrima*) were found in disturbed habitats like abandoned rice fields. Despite their quite different breeding strategies, *O. breviligulata* and *O. perennis* subsp. *barthii* were often sympatric. But they showed a tendency to habitat segregation as the former species grew at a lower zone (some 10 cm or less) than the latter species.

O. punctata seemed to be divisible into two ecotypes, one adapted to marshes shaded by trees, and the other adapted to open habitats and growing often sympatrically with *O. breviligulata*.

c) Cultivated rice: There were different types of rice fields which were divisible into upland, rainfed lowland, and river flood plain types, in addition to modernized irrigated fields. The rice fields of peasant farmers were generally undulated but not sectioned by dykes. In a majority of those fields, the two species, *O. sativa* and *O. glaberrima*, were mix-planted in varying proportions. Although governmental agencies recommend planting *sativa* cultivars, many farmers recognize that the traditional *glaberrima* rice is more tolerant to drought, submergence, and other stresses. The farmers rather willingly accept mixing of the two species and their

different varieties.

Irrigable rice fields have been open in some places in the semi-arid inland. There, we learned that the semi-dwarf improved Indicas of *O. sativa* introduced from Asia were generally inadaptive. This is possibly because the varieties selected for high yielding potential under a good cultural management have not been selected for tolerance to water stresses.

d) Character variations within rice populations: The seed samples collected from the fields showed that the populations were highly polymorphic in visually distinguishable characters. Evaluating the diversity in seed type by the amount of information ($H=-\sum p_i \log_e p_i$), we found that in the fields where *glaberrima* and *sativa* were mix-planted, the diversity within *glaberrima* populations was highest when the relative frequency of *glaberrima* plants was 50–60 percent. Its paraboric regression line on the relative frequency was highly significant. A similar trend was also found for *sativa* populations. We found further that the within-population diversity was in both species correlated with species diversity in the field which was estimated (also by H) from percent biomass for each of coexisting species (rice and weed). These relationships suggest that the complexity of the biotic emvironment as shown by species diversity plays a role in maintaining polymorphisms in rice populations. It was also suggested that the temporarily changing water conditions and microtopographic undulations in the field impose a pressure of disruptive selection on the rice populations and plant community as a whole and result in greater species and genotypic diversity. The interrelationship found between genetic variation within rice populations and community structure, though the dynamics are difficult to elucidate, may be of some use in considering work for the conservation of genetic resources.

e) Other crops than rice: In addition to rice, sorghum, millet, pearl millet, maize, cowpea, groundnut, cotton, etc. are grown in the rainy season. In many places, two or more crop species were found to be mix-planted. According to a report from the Institute of Agricultural Research, Ahmadu Bello University, intercropping in certain conditions was advantageous in increasing total yield. In cowpea, the presence of cereals in the field reduced pest damages when no pesticide was sprayed.

The cowpea seeds obtained from local markets were various in size and color pattern in the same manner as were rice seeds. Presumably, both genetic heterogeneity in a species and species mixing play similar roles in

increasing the biotic complexity of the community and serve as stabilizing mechanisms. Attempts of agricultural modernization by monoculturing a single crop may encounter with various hazards unless the agro-ecosystems are carefully investigated and managed.

The Breeding Behavior of *Oryza perennis* Populations Observed in Thailand

H. I. OKA and Y. SANO

We have been working on variations in the breeding systems (Oka and Morishima 1967, Evolution **21**: 249), adaptive mechanisms (Oka 1976, Evolution **30**: 380), and the pattern of resource allocation (Sano and Morishima 1977, Ann. Rep. **27**: 92) of a wild rice species, *Oryza perennis* Moench. These investigations concern the variation in perenniality among strains of this species. But it has remained unwarranted if the populations actually exhibit perennial or annual habit in their natural habitats and how the proportion of sexual and asexual propagations is. To confirm this point, Oka has observed eight populations of *O. perennis* in Thailand in the early part of the rainy season (19–28 June 1976) when the wild rice plant had a height of 20–50 cm.

The sites selected for observation were waste lowlands between road embankment and rice field. Such places are regularly disturbed by man in accordance with the management of rice field, and are inundated for several months as the water level rises in the river. At the time of visit in June, most of the sites were still dry or shallowly flooded. At each site, 1-m^2 quadrats were set down, and each *O. perennis* plant was dug up to examine the underground part as to whether it had sprouted from a stem node of an old plant (ratooning) or from a seed. The result showed that one population consisted wholly of seedlings, 5 consisted of ratooned plants, while two others were mixtures of both. The seedling population had a larger number of buried seeds than the others. It was in a temporary swamp parched in the dry season, while ratooned populations were found in more deeply inundated sites. This suggested the role of water stress in conditioning the propagation behavior.

The plants raised from the seeds of these populations were observed for "reproductive effort" (percent seed weight to total dry-matter weight) and other various attributes of adaptive mechanisms (by Sano). The result

proved that the seedling population was of the annual type and the ratooned populations were of the perennial type, while those performing mixed sexual and asexual propagations were of intermediate perennial-annual type. An observation of isoenzymic variations controlled by alleles at two loci (Px_1 and Acp_1) indicated that the populations showing mixed breeding behavior were highly polymorphic and heterozygous. One of them consisted of 9 separate clumps which differed in characters. Such populations should have a high evolutionary potentiality. It was suggested that the wild progenitor of *O. sativa* could be such intermediate populations of *O. perennis*.

A Competition Experiment between Oryza perennis Strains in Greenhouse

Hiroko MORISHIMA

To observe how wild rice strains compete each other through their vegetative growth and reproduction, three strains, i.e., W120 (a perennial type from India), W593 (a perennial type from Malaya), and W630 (an annual type from Burma) were tested for their interactions. They were planted in pure stands and in 1: 1 mixtures of all binary combinations at 3 densities (20–×20 cm, 10–×10 cm, and 5–×5 cm), in 1976 summer in a concrete bed filled with newly prepared garden soil in a greenhouse. After seed shedding, the above-ground part of the plants were harvested for recording several character values, and a half of each plot was cultivated with a hoe. Then, the concrete bed was not watered for 6 months until irrigated in 1977 May. The numbers of buried seeds, seedlings, and ratooned plants were recorded in the second (1977) season.

The effect of competition as shown by the changes in performance of the first generation plants exhibited a circular order of relationships among the three strains, that was –W630<W120<W593<–. Contrary to expectation, the competition effect was stronger in widely spaced planting than in dense planting. The survivorship (percent ratooned plants in the second season) was 40% for W120, 84% for W593, and 2% for W630. The mean number of buried seeds per soil sample (10–×10 cm, to a 5 cm depth) was 4.5 for W120, 7.9 for W593, and 23.3 for W630. In the second season, the annual strain (W630) produced a much larger number of plants than the perennial strains (W120 and W593). The cultivated half of the plots had larger number of seedlings than the other half.

The weeds in the first season, which arose from their buried seeds in the soil, significantly differed in the number of species as well as in the total number of plants according to the strains of *O. perennis* planted. One of the purposes of this experiment was to learn to what extent the conditions in natural habitats can be simulated in a greenhouse. Many difficulties were, however, experienced for achieving this purpose.

The Inheritance of Copper Tolerance in a Hybrid Population of Rice

Hiroko MORISHIMA

An *Oryza sativa* strain sensitive to copper (108, an Indica from Taiwan) and an *O. perennis* strain highly tolerant to copper (W106, an annual type from India) were crossed, and their F_4 lines, 30 in total number, were tested for copper tolerance in gravel culture (with cultural solution containing 0 and 5 ppm *Cu*). Copper tolerance was shown by toxic-normal ratios in performance which was obtained by snythesizing measurements for leaf-stem dry weight and three other characters. The susceptible parent (108) gave a ratio of 80%, while the tolerant parent (W106) gave 190%, and the values for F_4 lines were distributed between the parental values. The parent-offspring regression (heritability) was estimated to be 0.36 (significant at 5% level) by comparing the data with those from a similar test of F_3 lines (recorded in 1975).

In general, the plants collected from the field show a tendency to "trade off" or negative association of tolerance with the performance in normal condition. But the hybrid-derived lines presently tested showed no such trend. The absence of trade-off was also recognized in a hybrid population of barnyard grass.

Measurements of copper content in the leaf-stem and root of the plants in copper-treated plot revealed significant differences due to lines. But the copper content and copper tolerance were not correlated among the lines. In contrast, in barnyard grass, copper content in the above-ground part of the plants was negatively correlated with copper tolerance. It seems that genes controlling various physiological processes regulate the expression of copper tolerance in plants.

Copper Tolerance and Competitive Ability of *Alopecurus aequalis* Strains

Hiroko MORISHIMA

Strains obtained from copper polluted and unpolluted control fields in Gunma and Yamanashi Prefectures, 23 in total number, were tested for copper tolerance. The seed of each strain was sown in pots (1/50 m^2) filled with soils containing 0 and 250 ppm copper, with two replications. As reported previously (Ann. Rep. **27**: 90), their copper tolerance was evaluated on the basis of viability in copper-containing soil as compared with that in control soil, after the plants were partly uprooted by frost needles in winter.

Strains from polluted fields tended to have higher viability than those from control fields when they were subjected to frost needles in copper-containing soils, and the heritability of viability shown by parent-offspring regression was 0.34 (significant at 5% level). Unlike the cases in barnyard grass and other summer annuals, the comparison of performance between the plants in toxic and normal soils did not seem to be useful for evaluating copper tolerance in this species. Tolerant strains with a high winter viability showed a high copper co:tent in the root, but showed no such trend as to the copper content in the leaves and stems.

Further, the 32 strains were tested for their competitive response to mix-planted milk vetch (*Astragalus sinicus*) in pots filled with normal soil. The competitive ability against milk vetch showed a negative correlation with copper tolerance shown by winter viability ($r=-0.50$, significant at 5% level). This serves as an example of "trade-off".

The Self-persisting Ability of Soybean Hybrid Lines in Semi-natural Conditions

Hiko-Ichi OKA

Glysine soja, the putative wild progenitor of cultivated *G. max*, naturally occurs in abandoned fields on the campus of this institute. It was found that if the seed was scattered on a grassland, a new population was established there and persited several years. In contrast, the cultivated soybean can not exist if not seeded by man. To look into the difference in self-persisting ability, two soybean cultivars, Chung-Hsing 2 and 3 (selected from hybrids for wide adaptability at Taichung) were crossed with the pollen grains of a wild strain obtained from the campus. The F_2 plants raised in

the 1975 season were observed for various characters and 30 plants showing different combinations of traits distinguishing the wild from cultivated plants were selected. The characters recorded were vine-climbing on bamboo sticks, pod twistedness, pod dehiscence, single seed weight, etc.

The F_3 lines were sown at three sites (A: experimental field, B: abandoned field, and C: grassland along the edge of a field) in April 1976. At each site, a line was represented by 16 plants raised in a 1-m^2 plot with 1.5 m spacing from the neighboring plots. At sites A and B, a 1.8 m bamboo stick was stood beside each plant to let it climb up. After recording characters, the plants were left in the field and were allowed to shed seed. Then, the field was left untouched and the number of plants naturally growing in the 1977 season was counted for each line.

The result showed that the number of self-seeded plants per m^2 was 0 to 720 (702 for *G. soja*) at site A, and 0 to 293 (552 for *G. soja*) at site B. At site C, a 0 to 100% range of coverage by the soybean plants was observed among the 30 lines though the number of plants was not recorded. The self-persisting abilities thus recorded at three sites were intercorrelated indicating that they were genotypically controlled. Further, the lines were planted with a 7 m spacing in 1977 to measure the distance of seed dispersal. How the abilities of self-persistence and seed dispersal are associated with the characters recorded will be examined by analysing the 1978 data.

Interactions among Five Summer Annual Weeds

Hiroko MORISHIMA

To observe the pattern of interactions among different weed species, this experiment was conducted with 9 strains belonging to five species of summer annual weed, i.e., *Erechtites hieracifolia* (Compositae, symboled D), *Arthraxon hispidus* (Gramineae, K), *Setaria viridis* (Gramineae, E), *Digitaria adsendens* (Gramineae, M), and *Echinochloa crus-galli* (Gramineae, H). The 9 strains were tested in pure and mix (1: 1 with a tester) stands; a K and a M strain were used as the testers. The seeds of these species collected in the fall months of 1976 were sown on cultivated field plots (each 1 m^2) with four replications. Starting one month after seeding, two of the four plots were walked over 5 times a week for three months, while the other two plots were left untouched as the controls.

The number of plants and dry matter weight per unit area, as well as the dry weight of greatest and smallest 5 single plants were recorded at seed maturity by harvesting the above-ground part of the plants. The data indicated first that the effect of walking over markedly differed according to species. The number of D plants sharply decreased due to walking over, but M showed no change in number. Second, the effect of mix-planting on survivorship also differed according to species combination and walking over. In terms of dry matter weight per unit area, M was the strongest competitor while D was the weakest in the control plots. In the walked-over plots, E was as strong as M in competition. Thirdly, the difference in dry weight between the greatest and smallest plants would be an expression of phenotypic plasticity. This difference was greatest in K and smallest in D, and tended to be larger in pure than in mix-planted plots.

Variation in Competitive Ability among *Oryza perennis* Strains

Yoshio SANO and Hiroko MORISHIMA

With the view to investigate the role of competition in adaptive strategy of wild rice, a mix-planting experiment of *Oryza perennis* strains was carried out. Thirty four *perennis* and two *sativa* strains were tested in mix-planting with a test-strain which was an Asian annual type of *O. perennis* (W 107). Dry plant weight at maturity and several other characters were recorded for each strain. The competitive ability of a strain was shown by $(X_{12}-X_{11})-(X_{21}-X_{22})$, where X_{11} and X_{12} stand for dry plant weight of strain 1 in pure and in mixed stand with 2, etc.

Among Asian *perennis* strains, the annual and intermediate annual-perennial types tended to have higher competitive ability than the perennial type. The American strains had a low competitive ability. A positive correlation existed between the competitive ability and "reproductive effort" (proportion of seed weight to total plant weight).

Based on covariations between competitive ability and other growth measurements, path coefficients were computed to estimate their contributions to the competitive ability. About 68 percent of the variance of competitive ability could be accounted for by three measurements taken from pure stands, density response (coefficient *a*, in the foregoing report), dry plant weight at 93 days after germination, and percent plant cover at 70 days.

A simillar experiment with an *O. sativa* strain (Taichung 65) was reported

last year (Ann. Rep. 27: 92). The positive association between competitive ability and reproductive effort found from the present experiment was not in the experiment conducted last year. The competitive ability with a *sativa* tester showed a negative correlation with reproductive effort. This indicates that the competitive response of *perennis* strains greatly differ according to the competitor. Presumably, this difference comes from different growth patterns and plant types as were also observed among the wild rice strains.

The Density Response of *Oryza perennis* Strains

Yoshio SANO

It is an established principle that the effect of standing density on plants is represented by $WN^a=K$, where W is mean single plant weight, N is density or the number of plants per unit area, K is a constant, and a is a coefficient of density response. To investigate the variations in density response, 23 strains of *Oryza perennis* (showing a perennial-annual continuum) and 2 *O. sativa* strains were tested at three density levels. The seedlings of each strains were planted at the rate of 2, 4, and 16 plants per pot (1/50 m^2), with 2 replications. At the maximum tillering stage (93 days after planting), the plants were harvested and were measured for the dry weight of leaves and roots. From the data, the a values were computed in respective strains. The a value for the above-ground part ranged from 0.51 to 1.10, and that for the root from 0.39 to 1.04 among the strains tested. In the value for root weight, the difference due to strains was statistically significant.

The density response as shown by a was positively correlated with the competitive ability of the strains with an annual strain of *O. perennis* (W 107 from India). Density response is an expression of phenotypic plasticity. The data indicate that the wild rice strains showing different breeding habits and adaptive mechanisms differ in density response.

Ecological Factors Controlling the Coexistence of *Drosophila simulans* and *D. melanogaster*

Masaoki KAWANISHI and Takao K. WATANABE

The two sibling species, *D. simulans* and *D. melanogaster*, are believed to coexist in nature sharing food and space. Using banana bait traps, *Dro-*

sophilla flies were monthly collected from October 1975 to November 1976 at three ecologically different sites in Mishima. A total of 11,276 specimens involving 29 species of Drosophilidae were collected. The relative abundance of *D. simulans* and *D. melanogaster* was apparently different at different sites; the former prefers a semi-domestic niche and the latter a domestic one.

The seasonal abundance of these species was compared. *D. simulans* peaked in population size twice a year, in July and September, while *D. melanogaster* showed only one population peak, in August. However, this seasonal separation of the sibling species seems to be a temporary and transient phenomenon, since the optimum temperature for *D. simulans* is the same for *D. melanogaster*. In fact, in some populations at Sapporo and Hiroshima, where *D. simulans* was very scare, *D. melanogaster* showed a clear bimodal seasonal activity, avoiding mid-summer. Thus, *D. melanogaster* inevitably restricts its breeding season to the mid-summer in Mishima to lessen the competition with *D. simulans*. For details, see Jap. J. Ecol. **27**, 279–283.

XIV. APPLIED GENETICS

Temperature Responses of Isogenic Lines with Different Earliness Genes of Rice

H. YAMAGISHI*, K. H. TSAI** and H. I. OKA

The number of days to heading of rice plants is controlled by various genes. It was concluded from repeated experiments that the major earliness genes in photoperiod insensitive Japonica cultivars were *E* (8th linkage group) which moved up the time of floral initiation about one week and *m* (independent of *E*) which intensified the effect of *E* (Tsai 1976, Japan. J. Genet. **51**: 115). A number of isogenic lines with different alleles at these two loci were established from the experiments. In Taiwan, the *m* gene produced stronger effect in the winter (first crop) season than in the summer (second crop) season, and it promoted heading in winter even when combined with *e*.

Seven isogenic lines carrying gene combinations *Em*, Em^+, *em*, and em^+ were tested in two growth chambers (one adjusted at 30°C in day and 25°C in night, and the other adjusted at 22°C in day and 17°C in night; both illuminated with about 4,000 lux light for 14 hours a day), an ordinary greenhouse (temperatures varying between 30° and 18°C, under natural

Table 1. Comparison of heading date among isogenic lines with different earliness genes

Genotype	Growth chamber		Greenhouse	Outdoors, summer	Outdoors, 11.5 h/day
	25°–30°	17°–22°	18°–30°	18°–30°	18°–30°
e m^+ (T65)	0 (120)*	0 (160)	0 (108)	0 (112)	0 (104)
$E^a m^+$	−14	−20	−41	−6	−7
$E^b m^+$	−15	−23	−41	−8	−7
$E^a m$	−27	−32	−50	−27	−14
$E^b m$	−18	−16	−33	−20	−10
e m	+8	+11	−3	−9	−1

* Number of days to heading in Taichung 65

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days in winter), and in two concrete beds (outdoor, one with 11.5 hour-days, and the other under natural daylength, in summer). The result of this experiment demonstrated that the heading-promoting effects of *E* and *m* markedly differed according to the environment. The mean number of days to heading of the four genotypes under respective conditions are given in Table 1, in which the number of days obtained for Taichung 65 (em^+) was taken as zero. In the growth chambers, particularly in the low-temperature chamber, the *m* gene appreciably retarded heading when combined with *e*, though it promoted heading in the greenhouse and field. This indicates that the effect of *m* is sensitive to temperatures. The epistatic effect of *m* on *E* was much stronger when *m* was combined with E^a (from northern China) than with E^b (from Hokkaido). The heading date of lines with E^am was retarded by short days.

Noise Sensitivity of Wild and Domestic Japanese Quails as Shown by the Fertility of Eggs*

Takatada KAWAHARA

When expose to noise, wild strains of Japanese quail showed a more remarkable depression in egg laying performance than the domestic control (Ann. Rep. **27**: 95). The effect of noise on the fertilities of four mating combinations between the wild and domestic birds was investigated this year. A total of 92 matings between the wild (W) and domestic (D) strains, W×W, W×D, D×W, and D×D, were examined. The birds at an age of 130 days were subjected to a noise treatment: a buzzer of 95 phons worked 12 times a day, once for one hour for 20 days. Egg fertilites were

Table 1. Effects of buzzer treatment on fertility of wild and domestic strains

Mating	No. of matings tested	Fertility (%)		
Female×Male		Pre-treatment	During noise treatment	Post-treatment
W×W	47	70.3	57.4	63.5
W×D	10	74.6	69.0	78.5
D×W	25	79.0	63.7	81.4
D×D	10	96.2	98.8	93.4

* This research was supported by a grant from the Environment Agency.

recorded in three periods of 9 days each, which were chosen on the basis of the time from fertilization to egg laying. Those were noiseless pre-treatment period, during noise treatment, and noiseless post-treatment period.

As shown in Table 1, the matings of W×W, W×D, and D×W had reduced fertilities in response to noise treatment. But the D×D mating showed no significant change. In other words, when the wild strain was used as either male or female parent, fertility was reduced. The wild strain used for this experiment had been reared in the cage for 10 generations after the capture of original birds in the field. The birds showed remarkable changes in productive and behavioral characters toward the domestic type as the result of "unconscious selection" in the cage (Ann. Rep. **24**: 72; Kawahara, T. 1976, Experimental Animals **25**: 351). Yet, they retained the higher noise sensitivity of the wild type as was demonstrated in this experiment.

Nitrogen Fixation in Rhizosphere of Rice Plant

Yukinori HIROTA, Taro FUJII, Yoshio SANO and Sinya IYAMA

In order to investigate the nitrogen fixing ability in paddy rice, the following experiment were carried out.

(A) Improvement of an assay system.

So far various assay systems have been reported to investigate nitrogen fixing activity of rice rhizosphere. The result of them showe dconsiderable time lag before the nitrogen fixing activity appeared, arousing a question whether the activity measured was due to a real nitrogen fixation of rice rhizosphere or not. In our system, that of an intact plant was assayed under anaerobic conditions as described below. Whole sample of an intact plant which included the soil and water or rice rhizosphere and the bulk of relatively undisturbed root system was enclosed in a vessel so that the exposure of rhizosphere to the air was minimized. Rigorous displacement of gas phase in the vessel with a gas-mixture of helium and acetylene (9: 1 in volume) was achieved two times repeatedly by a evacuation pump and flushing the gas-mixture up to 1 atm. Then the vessel was kept for 30 min. to equilibrate the system with the gas, then it was evacuated and the gas-mixture was flushed again. The vessel was incubated under the laboratory room light condition at 28°C and fractions of the gas phase were withdrawn as a function of time using syringe for assay. Amount of ethylene

produced from acetylene were measured with a gas-chromatography.

Considerable activity of acetylene reduction was found in certain strains of rice. The production of ethylene from acetylene proceeded linearly and no time lag of the reaction was observed. When acetylene was not added to the system, no ethylene was produced. Soil without plant showed no detectable acetylene reducing activity during 6 hours of incubation and later small but significant amount of ethylene production was observed. Aerobic condition for the acetylene reduction assay showed lower activity than anaerobic condition. Thus, our assay system should be considered to measure the nitrogen fixing activity in rice rhizosphere properly.

(B) Genetic variation of nitrogen fixation.

The nitrogen fixation activity of rhizosphere of 50 strains of rice was measured. The strains used were those collected from various places in the tropical Asia and preserved at the Genetic Stocks Center in this institute. The plants were grown in pots until they reached to the flowering stage and the activity of these intact plants, two each a strain, was measured under the assay condtions described above. A wide range of variation was observed among strains (Table 1). Moreover, strong correlation (r= 0.895, significant at the 1% level) was observed between root weight and nitrogen fixing activity within a strain. Namely, the larger the amount or root per unit area, the higher the activity was observed. This means that existence of rice root is apparently essential in nitrogen fixation by bacteria in paddy field.

Table 1. Variation in acetylene reducing activity of 50 rice strains

	ethylene produced (n mole/plant/hour) 0–200–400–600–800–1000–1200	Total number of strains observed
No. of strains	13 23 8 2 3 1	50

(C) Rice-bacterial association.

Seeds of a rice strain which exhibited a high activity of nitrogen fixation were sterilized and seeded on sterilized soil, and grown under the germ-free growth condition. The activity of nitrogen fixation was measured. No activity of acetylene reduction was detectable on the sterile plant, suggesting the presence of microbial involvement in the activity. An anaerobic bacterium, rod shaped, spore formers at the cell terminus, gram positive and biotin require as a growth factor was isolated. It is likely to

be a *Clostridium*. It is known however that from a single soil sample one can isolate many species of soil bacteria capable of fixing nitrogen, and it is difficult to judge whether or not this bacterium is responsible for the nitrogen fixation of rice rhizosphere. A systematic examination of nitrogen fixing bacteria in the rhizosphere of rice in our experimental field are in progress.

Analysis of Genes Controlling the F_1 Sterility between *Oryza sativa* and *O. glaberrima*

Yoshio SANO

Experiments on the genetic basis of the F_1 sterility between two cultivated rice species, *O. sativa* and *O. glaberrima*, have been continued since 1966 (Ann. Rep. **24**: 67, **25**: 85, & **27**: 80). In the previous work, an Indica strain (108 from Taiwan) was used as the *sativa* parent, and from its cross with a *glaberrima* strain (W 025 from Sierra Leone), isogenic F_1-sterile lines with the genetic background of either parent were established and were used for crossing experiments. The result proved that the F_1 sterility was at least partly controlled by alleles at a certain locus which produced a particular sporo-gametophytic interaction in heterozygote (Ann. Rep. **27**: 80).

A following-up experiment was initiated in 1975 by using a Japonica strain, T65, as the *sativa* parent, and W025 as the *glaberrima* parent in the same manner as in the preceding experiment. The backcross generations so far observed (up to B_6) showed a different pattern of segregation from that observed before. Namely, out of 52 B_5F_1 and 23 B_6F_1 (75 in total), 33 were semi-sterile and 41 were fertile, giving a 1 : 1 ratio. Since two of the lines showing the same pattern of segregation had the cytoplasm of *glaberrima* parent, the possibility that the sterility is controlled by gene-cytoplasm interaction may be rouled out.

This type of segregation has been observed throughout backcrossing experiments between distantly related *sativa* varieties (Oka 1974, Genetics **77**: 521). On a series of evidence, the partial F_1 sterility between *sativa* cultivars was attributed to duplicate gametic lethals. Possibly, a similar genic system is involved in the F_1 sterility between the two rice species. Yet, it seems plausible to assume that various genic systems contol the F_1 sterility between two sibling species, and most dominant one is selected when the backcross method is used for isolating isogenic F_1-sterile lines.

Breeding of Isogenic Lines of Rice Carrying Gene Markers in Interchanged Chromosome Segments

Yoshio SANO

This work was initiated by Oka (Ann. Rep. **24**: 66), and has been continued for 11 years. Isogenic lines of Taichung 65 having different gene markers, respectively, were early established. The genes chosen as markers were *nl* (neck leaf), *bc* (brittle culm), *g* (long empty glume), *lg* (liguleless-ness), *gl* (glabrousness), *Rc* (brown pericarp), *wx* (glutinous endosperm), etc. The pleiotropic effects of these genes on metric characters were sometimes significant but trivial in general (Ann. Rep. **25**: 84). If these gene markers are involved in interchanged chromosome segments, the isogenic lines carrying them may be more useful testers for genic analysis. For this purpose, crosses were made between isogenic marker lines and isogenic translocation lines (Ann. Rep. **27**: 102).

This work is still under way, partly with the correlation of Dr. S. Sato of the Ryukyu University. So far, 7 lines carrying a marker in a translocated segment ($T2^{g}$, $T39^{g}$, $T44^{g}$, $T55^{g}$, $T52^{nl}$, $T54^{bc}$, and $T55^{lg}$) were isolated. In many of them, the recombination value between marker and the point of interchange was about 10% or less. These will be used for observing the genetic control of attributes of adaptive mechanisms.

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ABSTRACTS OF DIARY FOR 1977

January	24	241st Meeting of Misima Geneticists' Club
February	14	242nd Meeting of Misima Geneticists' Club
	23	125th Biological Symposium
March	9	243rd Meeting of Misima Geneticists' Club
	16	244th Meeting of Misima Geneticists' Club
	25	245th Meeting of Misima Geneticists' Club
April	18	126th Biological Symposium
June	1	246th Meeting of Misima Geneticists' Club
	22	127th Biological Symposium
August	29	247th Meeting of Misima Geneticists' Club
September	7	128th Biological Symposium
	9	129th Biological Symposium
October	5	130th Biological Symposium
	18	131st Biological Symposium
November	2	132nd Biological Symposium
	17	248th Meeting of Misima Geneticists' Club
	22	133rd Biological Symposium
	26	134th Biological Symposium
December	5	135th Biological Symposium
	16	136th Biological Symposium

FOREIGN VISITORS IN 1977

February	23	GROS, F., L'Institut Pasteur, France.
April	7–14	CROW, J. F., University of Wisconsin, USA.
	18–19	FITCH, W. M., University of Wisconsin, USA.
	22–23	AMBLER, R. P., University of Edinburgh, UK.
June 29–July	12	JONEJA, M. G., Queen's University, Canada.
September	6–7	SCHNEIDERMAN, H. A., University of California, USA.
October	9	STERN, H., University of California, San Diego, USA.
	13	CONSTANS, J., Centre National de la Recherche Scientifique, France.
November	8	SIDDIQUI, K. A., Atomic Energy Agricultural Research Center, Pakistan.
	23	NARAYANSWAMI, N., Commissioner for Sericulture, Government of India.
	23–24	FRAENKEL-CONRAT, H., University of Calfornia, USA.
	26	SPIRIN, A. S., Institute of Protein Research, USSR.
December	5	MAHER, V. M., Michigan State University, USA.
	5	MCCORMICK, J. J., Michigan State University, USA.
	16	ARLETT, C. F., University of Sussex, England.

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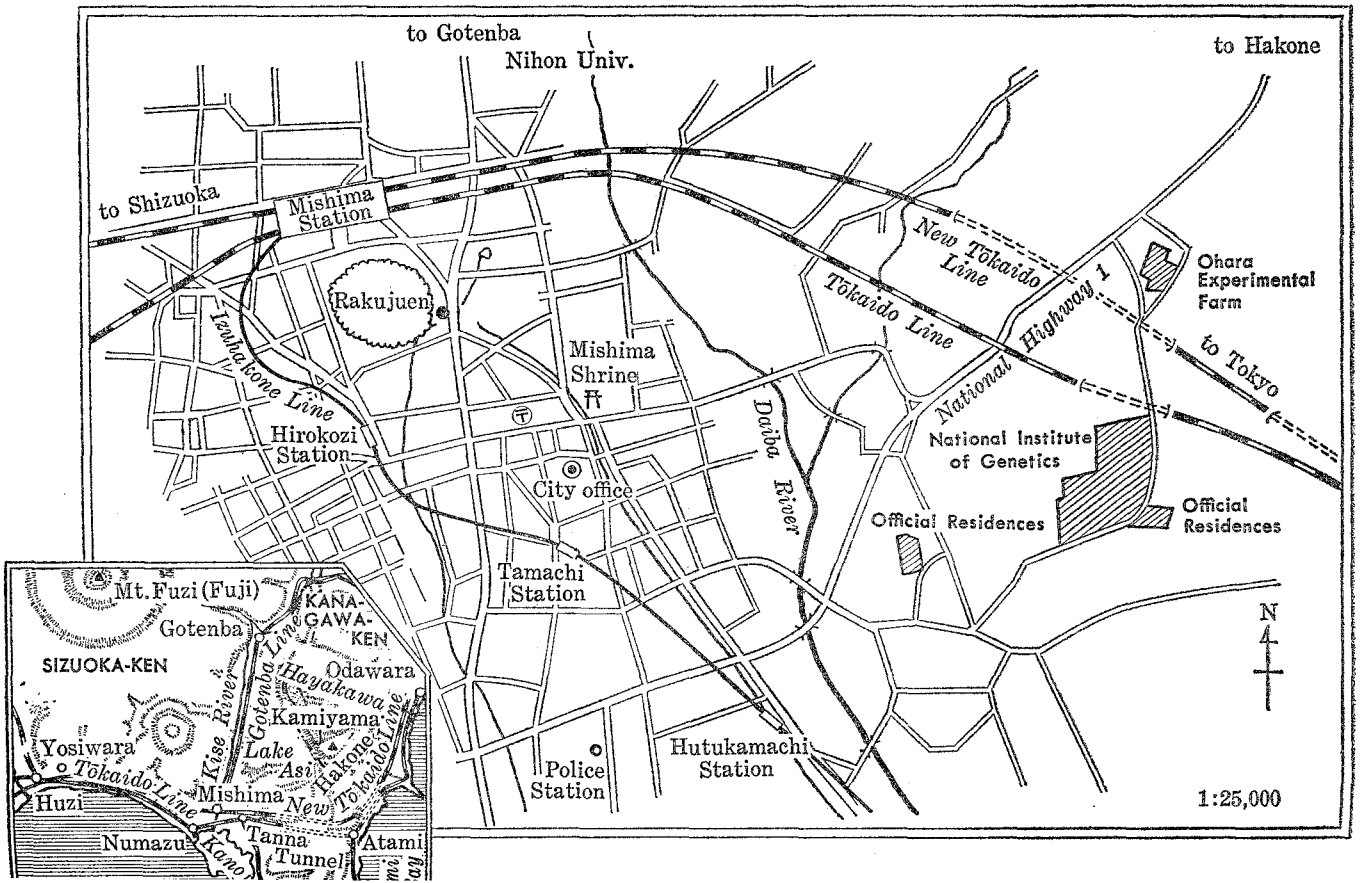
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